Cross-feeding of Vitamin B_1, purines, and their biosynthesis intermediates in *Escherichia coli* co-cultures

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by

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To

my parents, Madhuri and Ravindra

who have always backed me…

DECLARATION

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Dr. Amrita B. Hazra
(Supervisor)

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"Help will always be given at Hogwarts to those who ask for it"

- Albus Dumbledore, Harry Potter and the Chamber of Secrets

(J. K. Rowling)

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Cross-feeding of Vitamin B_1, purines, and their biosynthesis intermediates in *Escherichia coli* co-cultures

Abstract

A diverse array of microorganisms inhabits majority of the ecosystems on Earth. These microbes are often auxotrophic for essential nutrients and thus are engaged in a dynamic exchange of metabolites with other members in their community. Such a cross-talk gives rise to an intricate web of interactions, which shape the structure and function of microbial communities.

The complexity of multiple interactions obscures our ability to understand how a microbial community is formed and sustained, and how its members proliferate to reach a stable composition. Thus, building a synthetic microbial community with a set of defined microbes and proposing hypotheses regarding the interactions, followed by testing which ones hold true using tools in microbiology and analytical biochemistry is a systematic approach to initiate a probe into more complex communities. In this thesis, we analyzed the exchange of Vitamin B_1 (thiamin), purines, and their biosynthesis intermediates in microbial communities. In *Escherichia coli*, thiamin biosynthesis intermediates 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) and 4-methyl-5-(2-hydroxyethyl)thiazole (THZ) are synthesized *de novo* in two distinct branches of the pathway. HMP is synthesized by ThiC in a single step rearrangement reaction from 5'-phosphoribosyl aminoimidazole (AIR), which is also an intermediate shared by the purine biosynthesis pathway. AIR is synthesized by PurM and is further committed to purine biosynthesis by the enzyme PurK. THZ is synthesized by ThiG, which is coupled with HMP by ThiE to form thiamin. To analyze the molecular cross-talk between thiamin and purine biosynthesis pathways, we designed a system based on synthetic co-cultures of *E. coli*.

Towards this goal, we created single gene knockout mutants *ΔthiC*, *ΔthiE*, *ΔthiG*, *ΔpurK*, and *ΔpurM* of *E. coli* str. K-12 substr. MG1655. We observed that *ΔthiC-ΔthiE* and *ΔthiC-ΔthiG* co-cultures of *E. coli* survived in minimal media in the absence of exogenous thiamin, whereas the *ΔthiE-ΔthiG* co-culture did not. Further analysis confirmed that the survival of these co-cultures is based on the exchange of HMP and thiamin, whereas THZ exchange in them is limited. We also

co-cultured *ΔpurK* and *ΔpurM* mutants with these three thiamin mutants and observed that only the *ΔthiC-ΔpurK* co-culture survived in the minimal medium in the absence of external purine and thiamin supply. These results further support the exchange of HMP in co-cultures of *E. coli* mutants.

We also quantified the ratio of mutants in two- and three-membered co-cultures of thiamin mutants, and observed that it changed in the absence of exogenous thiamin, suggestive of a cross-talk. The percentage of the *ΔthiC* strain under these conditions increased and stabilized to constitute about 80% of total cells, which could support a population of other cells $1/4^{th}$ of their own population size. But in the presence of thiamin, their ratios did not deviate much from the starting ratio, indicating that the mutants did not interact. We also visualized the spatial patterns generated by these co-cultures in the presence and absence of thiamin supplementation, and observed that the mutants co-localized in the absence of thiamin, indicating their need for interactions based on metabolite exchange.

The findings from this thesis highlight the importance of exchange of essential metabolites in shaping and stabilizing the interactions in microbial communities. Such studies can be used to determine the underlying principles of specific molecular-level interactions and to design synthetic communities for various biotechnological applications.

Chapter 1: Interactions in microbial communities in diverse natural habitats and synthetic systems

1.1 The world of microbial interactions

Microorganisms from all domains of life such as viruses, bacteria, archaea, unicellular fungi, and protists occupy almost every ecosystem on Earth in some form or another. A group of microorganisms in which the microbes are closely associated and interact with each other and with the other members of the niche they occupy is termed as a microbial community or consortium in that system[1]. Microbes in such communities often exchange metabolites such as vitamins, amino acids, nucleobases, metal chelating siderophores, electron donors and acceptors with other microbial and eukaryotic members in their surroundings[2–8]. Such an exchange of nutrients gives rise to a complex web of dynamic interactions within the ecosystem, either beneficial or detrimental to a particular species, and leading to spatiotemporal changes in the community composition and structure, subsequently affecting the processes in the habitat[1–3,9].

Analysis of microbial communities is of rising interest in order to identify emergent metabolites such as antimicrobials, to improve industrial yields of products produced by microbes, to analyze bacterial interactions for developing strategies against pathogens, to develop treatments against diseases such as faecal transplants, and also to understand types of biological interactions in microbial communities[2,10]. To achieve this, microbial interactions are studied either in the natural habitat of microbes, or in semi-natural/ semi-artificial systems and synthetic communities set up in a laboratory. With advances in technology, a variety of methods are being developed today to analyze these interactions in more details, such as chemical methods for metabolite identification, physical devices for setting up cultures, computational modeling and omics technique based data generation, and microscopic analysis to visualize interactions[11]. Using these tools, scientists are trying to understand the types of interactions that shape microbial communities, some of which are ecological, some are based on spatiotemporal arrangement, and others are based on exchange of metabolites[1]. These methods have also identified a diverse range of metabolites

that are being exchanged in microbial communities, ranging from large, complex molecules such as carbohydrates to even small, sub-atomic particles such as electrons[3,7,12].

In this chapter, we discuss the salient features of microbial communities and their analysis, enlisted in Table 1.1 below. We also discuss the literature available on exchange of vitamins and their biosynthesis pathway intermediates, especially for vitamin B_1 (also known as thiamin) in microbial communities, and our strategy to analyze their exchange in synthetic co-cultures of *Escherichia coli* thiamin auxotrophs.

Why study microbial interactions	1. Production of antimicrobial compounds and developing strategies against infections 2. Improving production of industrially-relevant secondary metabolites 3. Analyzing ecological and evolutionary interactions among microbes
Types of microbial communities being studied	1. Natural microbial communities – soil microbial communities maintain nutrient levels in soil and improve plant health, aquatic microbial communities and nutrient cycling, healthy skin and surface microbiota protects against pathogens, healthy microbial communities in digestive tracts of eukaryotes help in digestion of food and provide innate immunity 2. Semi-natural or semi-synthetic microbial communities 3. Artificial or synthetic microbial communities
Methods used to analyze microbial communities	1. Equipment used – microfluidic chambers, iChip with semipermeable membranes, bacterial microcontainers made with multiphoton lithography, scanning electrochemical microscopes 2. Experimental chemical techniques – high performance liquid chromatography (HPLC), LC coupled tandem mass spectrometry (LC-MS/ MS), matrix assisted laser desorption/ ionization coupled with imaging mass spectrometry (MALDI-IMS), Nanospray desorption electrospray ionization (Nano-DESI) MS 3. Computational tools and visualization techniques – high throughput sequencing, metagenomics, metatransctiptomics, proteomics,

	metabolomics, computational models and tools such as those for flux balance analysis, confocal microscopy to visualize spatial patterns, electron microscopy to visualize specific structures such as nanotubes
Types of interactions in microbial communities	1. Positive and neutral interactions – commensalism, mutualism or cooperation which can be homotypic or heterotypic (kin discrimination/ partner choice and kin fidelity/ partner feedback fidelity), cheaters arising in cooperative communities due to mutation or migration leading to the 'tragedy of the commons' or 'adaptive race', altruism 2. Negative interactions – amensalism, competition, predation, cannibalism, fratricide
Types of metabolites exchanged among microbes	Carbon sources, amino acids, nucleobases, siderophores, minerals, vitamins, electrons, antimicrobial compounds

Table 1.1 Types of interactions in a microbial community, the reasons for which they are studied, and the approaches used to analyze them

1.2 Why study interactions within microbial communities

An interacting consortium of microorganisms produces a huge variety of metabolites, some of which might be used as nutrients by others, whereas some are toxic to others. Production of such antimicrobial compounds is often organism- and interaction-specific, and might not occur in the absence of the microbes being harmed by them, thus necessitating the analysis of microbial communities[3]. Such studies help develop newer strategies and antibiotics against drug-resistant pathogens. Understanding the role of native microbiota in human health also helps in advancing techniques in modern medicine. Microbial interactions also lead to production of secondary metabolites, many of which are products that are used by us on a day-to-day basis[10]. Apart from this, analyzing the ecological and evolutionary interactions within microbial communities can help gain insights into newer biological phenomena, furthering our understanding of basic biology[1].

1.2.1 Production of antimicrobial compounds and developing strategies against infections

Microorganisms in a community often compete with each other for nutrients and space, at times, producing substances that inhibit the growth of other microbes in the consortium. For example, *Bacillus subtilis* produces a surfactant called surfactin that allows formation of aerial hyphae in it, but inhibits hyphae formation by *Streptomyces coelicolor*, a bacterium that co-occurs with it in soil[13]. It has also been observed that certain bacteria produce non-toxic secondary metabolites, which do not affect growth, but rather hamper the phenotypes regulated by quorum sensing in other bacteria[14]. The accidental discovery of penicillin, an antibacterial compound produced by the mold *Penicillium chrysogenum* which was shown to inhibit the growth of *Staphylococcus aureus* and which is used extensively for treating infections today, signifies importance of studying such interactions[15]. An antibacterial compound called andrimid, produced by certain marine bacteria, has also been shown to inhibit the growth of pathogenic *Vibrio cholerae*, which can be used as a treatment strategy against its clinical isolates[16].

Analyzing interactions between pathogens and native microbes that co-occur in infections can also significantly increase our understanding of disease pathobiology, allowing development of newer strategies to combat infections. For example, *Pseudomonas aeruginosa* helps *Staphylococcus aureus* to develop aminoglycoside resistance by producing 4-hydroxy-2-heptylquinoline-N-oxide (HQNO), forming resistant, small colony variants of the latter, similar to those in cystic fibrosis patients[17]. Yet another study of a 3D-printed co-culture with a *S. aureus* core surrounded by a shell of *P. aeruginosa* showed increased survival rate of *S. aureus* in the presence of ampicillin due to the production of beta-lactamase by *P. aeruginosa*[18]. Biofilms of pathogens that are difficult to get rid of are also studied to devise treatments against infections[19].

Native microbial inhabitants of a system are also used as a supplement to treat infections, termed as a probiotic treatment, which can be further improved by adding nutrients (prebiotics) that these microbes feed on[20,21]. Role of probiotics as an alternative treatment is getting increasingly popular against a variety of diseases, including travellers' diarrhoea, irritable bowel syndrome, ulcerative colitis, *Helicobacter pylori* infection, atopic dermatitis and several respiratory tract infections[22,23]. The bacteria that have been used as probiotics for improving gut health include native gut inhabitants such as *Lactobacillus acidophilus, L. casei, L. lactis,*

Bifidobacterium bifidum Enterococcus faecalis, and bacteria from yoghurt, *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*. The native microbiota of gut and the invading pathogens have also been co-cultured synthetically to gain more insights into their interactions with the intestinal wall and their ability to modulate immune system[24]. Efforts are also being made to co-culture the aerobic cells of human gut with its obligate anaerobes such as a human oxygen bacteria anaerobic (HoxBan) system developed using the Caco-2 cell line and an obligate gut inhabitant *Faecalibacterium prausnitzii*[25].

1.2.2 Improving production of industrially-relevant secondary metabolites and food products

Microbial co-cultures are also used for industrial production of biomolecules which is otherwise difficult, due to problems such as high starting material costs or technology limitations. For example, yield of 2-ketogulonic acid, a vitamin C precursor produced by *Ketogulonicigenium vulgare*, was increased by co-culturing it with another bacterium *Bacillus megaterium* that releases purines which are used by *K. vulgare* to avoid supplementing a large quantity of purines[26]. In yet another study, a synthetic co-culture of *E. coli* strains was used to improve the yields of 3-aminobenzoic acid by almost 15-30 fold, thus avoiding the negative effects of engineering the entire pathway in one organism[27,28]. Also, techniques such as immobilization of co-cultures in a bioreactor bed have been used to improve production of certain compounds such as acetic acid[29]. In addition, cheese rind communities are being increasingly used to understand microbial interactions that shape structure and function of microbial communities[30–32]. Yogurt, yet another product consumed on a large scale, is produced by metabolite exchange between *Lactobacillus bulgaricus* and *Streptococcus thermophilus*[33,34].

1.2.3 Analyzing ecological and evolutionary interactions among microbes

Apart from the benefits mentioned earlier, microbial co-cultures are also studied to gain insights into the ecological and evolutionary processes that help develop their structure and function. One such study put forth the black queen hypothesis, which hypothesizes that an organism undergoes loss of genes involved in useful but leaky functions as long as there are other helper microbes in a community which help the former organism with these functions[35]. Here, the authors analyze the help provided by *Synechococcus* sp., which harbours the *katG* gene involved in reducing redox-active species, to members of *Prochlorococcus* sp. against resistance to

oxidative stress, which have lost the gene from their genome. This is akin to holding a Queen of Spades in the game of Hearts, which costs high penalty, similar to the metabolic cost associated with having *katG*, leading to reductive genomic evolution, also observed in other cases[36]. Yet another study showed that adaptive evolution of a cooperating community of *E. coli* strains involved in exchange of leucine and lysine over 100 generations lead to improved growth of the community[37]. But it also lead to increased interdependency such that the growth of individually evolved strains supplemented with amino acids was poorer than unevolved strains. Such phenomenon has also been observed for another *E. coli* community, wherein the cost for improving the community function was increased sensitivity to antibiotic stress during adaptive evolution[38]. Such studies reveal that biological systems are typically complex, and put forth the need to analyze even more microbial communities in detail.

1.3 Types of microbial communities being studied

In order to scrutinize the structure and function of microbial communities, it is preferred at times to analyze them directly by sampling from their natural habitat (Figure 1.1)[2]. At times, semi-synthetic or semi-natural systems are used to cultivate a portion of microbes from natural communities, while trying to maintain the overall community function. In other cases, entirely synthetic communities consisting of microbes that might not co-occur in nature are devised for improving their function.

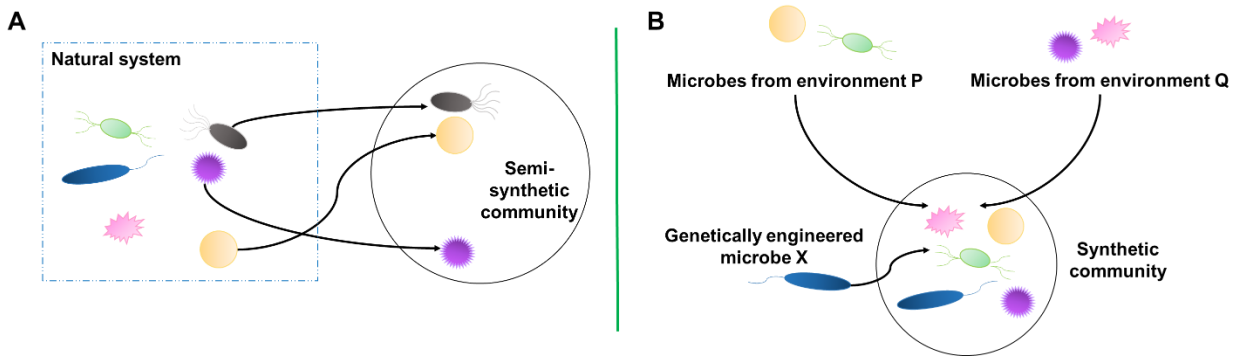

Figure 1.1 Types of microbial communities being studied. (A) A natural community comprising of all the organisms in the ecosystem and a semi-natural or semi-synthetic community consisting

of a subset of microbes from the natural community. (B) A synthetic community consisting of microbes from different environments.

1.3.1 Natural microbial communities

Microorganisms inhabit a wide range of natural ecosystems, including soil, marine and freshwater systems, air, and skins and guts of eukaryotes. Several of them can also survive under extreme and harsh conditions such as high pressure and heat, high pH, high radiation, low temperatures, and low to no oxygen. Presence of a healthy microbial consortium is essential to keep these ecosystems stable and functional.

1.3.1.1 Soil microbial communities maintain nutrient levels in soil and improve plant health

Most plants obtain water and a majority of micronutrients such as nitrogen, phosphorous, potassium, magnesium, iron and sulfur from the soil surrounding their roots. Microbial communities play a crucial role in releasing these nutrients in the soil[39]. Any environmental disturbances such as climate change, spread of plant pathogens, and changes in soil nitrogen and carbon content affect the community composition along with other factors like moisture and temperature[40,41]. The microorganisms closely associated with the plant roots (i.e. in the rhizosphere) supply various benefits to plants in exchange for carbohydrates, amino acids and space, which also reduces the genomic burden of the microbiome. Proteobacteria, Actinobacteria, Chloroflexi, Firmicutes, Ascomycota, Basidiomycota, Thaumarchaeota, Crenarchaeota, *Pythium*, and *Albugo* are some of the abundant bacterial, fungal, and archaeal phyla and protists in the soil as revealed by metagenomic analyses. Certain nodulating bacteria invade the root cells of leguminous plants by modifying their regulatory pathways, and multiply and grow in the root nodules, forming a symbiotic relationship with the host plant such as *Rhizobium, Bradyrhizobium,* and *Mesorhizobium*[42]. Interactions between plant-associated bacteria that might provide nutrients such as purines to plants under nitrogen deficiency are also being studied to improve crop yields[43].

Apart from the nutrient supply, the native microbiota of plants also helps in protecting the plants from a variety of plant pathogens. For example, *Ralstonia solanacearum* that causes bacterial wilt is enriched in the soil microbiota of tomato and tobacco[44,45]. On the other hand, beneficial microbes such as *Flavobacterium, Bacillus, Agromyces, Micromonospora,*

Mesorhizobium are enriched in the healthy, resistant plants as compared to the wilted, susceptible ones. Recruitment of microbes that produce antimicrobial and antifungal compounds, terpenoids and other volatiles is yet another strategy using which plants gain protection against pathogens[39].

1.3.1.2 Aquatic microbial communities and nutrient cycling

Aquatic ecosystems, among others, consist of picoeukaryotes such as green algae, haptophytes, stramenopiles, and dinoflagellates. Some microbes such as *Synechococcus* degrade complex polysaccharide molecules in the form of particulate organic matter, thus helping in biogeochemical cycling of nutrients[46]. Microbial consortia of organisms such as *Alcanivorax* and *Dietzia* help clean up the oil spills in the ocean[47]. In some cases, bacteriophages have been shown to lyse their bacterial hosts, thus releasing the nutrients which can be used by other microbes[48].

1.3.1.3 Healthy skin and surface microbiota protects against pathogens

Native microbiota occurring on the surface of aquatic organisms such as amphibians and fish has been shown to protect them from deadly pathogens[49]. Skins and surfaces of other land-dwelling eukaryotes such as animals and humans also show presence of a variety of microorganisms. For example, *Staphylococcus lugdunensis* produces an antibiotic called lugdunin that prevents colonization of nasal surfaces by *S. aureus*[50]. Microbes on surfaces of plant leaves are also shown to protect them from pathogens, though such interactions might depend on formation of spatial structures such as aggregates[51].

1.3.1.4 Healthy microbial communities in digestive tract of eukaryotes help in digestion of food and provide innate immunity

A variety of microorganisms are found to dominate digestive tracts of eukaryotes, some of which might be pathogenic and form biofilms. For example, members of oral microbial community such as *Actinomyces* sp., *Spirochaetes*, and *Streptococcus* sp. form corncob-like structures, eventually leading to diseases such as dental caries[52]. Microbial communities in human gut also play a role in digestion of complex polysaccharides in return for other nutrients[12]. In addition, members of gut microbiota such as *Lactobacillus* sp. and *Bifidobacterium* sp. have been shown to displace pathogens[23]. Native gut microbes have also been shown to accumulate drugs used for treating human diseases, leading to microbial cross-feeding and host-microbe interactions[53].

1.3.2 Semi-natural or semi-synthetic microbial communities

Several studies have been carried out using microbes isolated from their natural habitats in order to gain more insights into the mechanisms that operate in nature. In one such study, a device called iChip that has channels separated with semipermeable membrane was used to identify teixobactin, an antibiotic produced by microbes after interacting with other organisms in their natural environment[54]. Yet another study used two consortia of marine microbes to analyze exchange of vitamins in those communities[55]. Microorganisms that colonize cheese rind have also been analyzed to determine the nature of complex interactions among them[32,56]. Additionally, semi-synthetic microbial community from Drosophila gut has been studied to identify metabolites exchanged within it and its impact on host behavior[57]. Also, metabolite exchange among microbes from human gut has been analyzed, in some cases, even for drug assimilation by them[53].

1.3.3 Artificial or synthetic microbial communities

Different bacterial strains or species belonging to a same genus may not have the same phenotype, and this property can be used to study interactions of two strains or species involved using a co-culture. For example, exchange of nutrients among microbes has been utilized for large-scale production of hydrogen, 3-aminobenzoic acid, 2-ketogulonic acid, and many others[10,26,28,58]. Synthetic cultures have also been employed to analyze interesting mechanisms such as quorum sensing in which one species 'eavesdrops' on signals sent from other species, or understanding the role of spatial distance in microbial communities, or studying evolution of co-cultures[37,59,60]. For example, a study on artificial co-culture of *Chromobacterium violaceum and Burkholderia thailandensis* revealed an interesting mechanism in which the former species gains an advantage over the latter during competition in the co-culture by 'eavesdropping' on the quorum sensing signals of the latter[59]. This eavesdropping defined as intercepting the quorum sensing signals, conferred a growth advantage to *C. violaceum* in this community as the quorum sensing in both these bacteria is linked to the production of antimicrobials. Synthetic cultures have also been utilized to analyze adaptive evolution, one such example is the adaptive evolution study using co-cultures of *E. coli* K-12 BW25113 leucine and lysine auxotrophs[37]. The two strains were allowed to co-evolve and adapt over 100 generations and the amino acid exchange was studied. The study revealed that the rates of leucine and/or lysine uptake and release decided the growth rate, biomass

and survival of the two auxotrophs. Evolved cells from the co-cultures behaved differently in all these aspects when cultured singly or even as compared to their un-evolved counterparts.

1.4 Methods used to analyze microbial communities

With advances in technology, novel methods are being designed to gain a better understanding of the microbial systems[11]. Physical devices which allow culturing smaller volumes of microbes are being designed, along with chemical analysis techniques that help in identifying the exact nature of metabolites exchanged between microbes. Computational tools and models have been devised to identify the microbial interactions that can be studied experimentally, and visualizing such interactions has become easier with microscopic analyses of co-cultures.

1.4.1 Equipment used

Devices that have microfluidic chambers which allow the continuous flow of microbial culture volumes in microliter range are being increasingly used to analyze microbial interactions[61]. Such devices can be fitted with semipermeable membranes which allow only the flow of nutrients between the chambers that contain different microbial members, such that microbial communities that require optimal spatial distance can be analyzed[60]. Spatial segregation in such devices can also help in developing functions such as bioremediation in microbial co-cultures, which can only be stabilized after physical separation[62]. Devices like iChip which have similar membrane-separated channels can be transported back to the natural environment once the microbes to be analyzed are inoculated in them, forming a semi-natural system for analysis[54]. Bacterial microcontainers which are three dimensional structures of picolitre volumes formed by cross-linking bovine serum albumin with multiphoton lithography technique have been utilized to study biofilm formation and antibiotic resistance with cell numbers as low as 150 cells[18,63]. They have also been coupled with electrodes to form scanning electrochemical microscopes for measuring small, local concentrations of molecules in real time[64,65].

1.4.2 Experimental chemical techniques

Techniques such as high performance liquid chromatography (HPLC), liquid chromatography coupled tandem mass spectrometry (LC-MS/MS), and nuclear magnetic

resonance (NMR) have been utilized for segregating the mixture of microbial metabolites, identifying the metabolites with similar chemical composition based on their mass and confirming the nature of such metabolites[3]. A modified technology called matrix-assisted laser desorption/ ionization coupled with imaging mass spectrometry (MALDI-IMS) has been utilized to visualize metabolites produced by microbes in a single colony on a solid medium[66]. Nanospray desorption electrospray ionization (Nano-DESI) MS technique has been utilized to identify emergent metabolites arising due to presence of interacting microbes using a small capillary that constantly transports chemicals from the colonies to a mass spectrometer[8]. These techniques have facilitated large-scale metabolomic analyses of microbial communities, making it easier to identify novel molecules and interactions.

1.4.3 Computational tools and visualization techniques

High throughput sequencing of microbial genomes obtained from environmental samples has been employed to identify uncultivable microorganisms from natural ecosystems such as human gut[67]. Metagenomic, metatranscriptomic, proteomic and metabolomic approaches have also been utilized to identify the vast number genes and proteins actively coded by those genes, and to quantitate metabolites affected by microbial interactions[55,68–70]. Metagenomics has helped to identify novel variants of metabolic pathways in microbes[68]. It has also been utilized to identify homologs of proteins in various organisms. In addition, computational models have been developed to understand the nature of interactions in microbial communities and to identify and calculate metabolite fluxes[71,72]. Spatial patterns formed by interacting microbes have been visualized under confocal microscopes by labelling them with fluorescent proteins[73,74]. Electron microscopy has also been employed to visualize specific structures such as nanotubes formed by interacting microbes[75].

1.5 Types of interactions in microbial communities

Microorganisms in communities are constantly engaged in a multitude of interactions, some of which affect certain microbes positively, whereas certain others affect them negatively. Certain interactions can be neutral where one of the microbes neither benefits nor gets harmed by other microbes in the community. These interactions can then be further classified into subtypes based on the behaviour of different members of the community and other correlated phenomena.

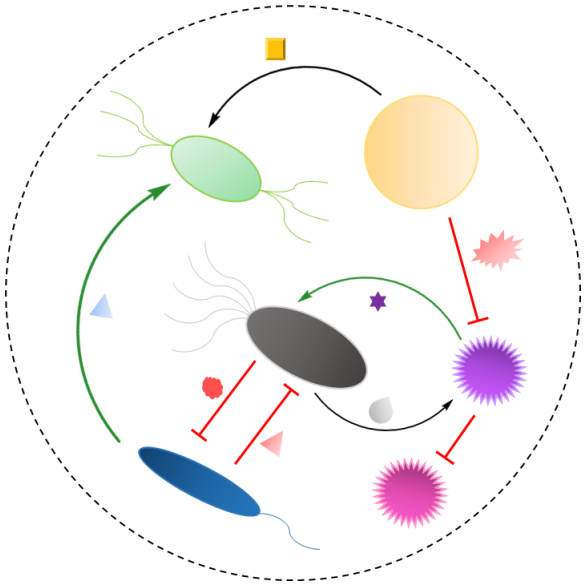

Figure 1.2 Types of interactions in microbial communities. Positive interactions: commensalism – yellow and green microbes, cooperation – between lavender and gray microbes. Negative interactions: amensalism – yellow and lavender microbes, competition – gray and blue microbes, fratricide/ cannibalism – lavender and pink microbes that are siblings.

1.5.1 Positive and neutral interactions

The positive interactions between two organisms that benefit one of them but do not affect the other are called commensalism[71]. But when both microbes involved in interactions benefit from each other, they are defined as mutualism or cooperation[12]. Cooperative interactions can be either homotypic (between individuals of same species) or heterotypic (individuals of different species), and arise due to either kin discrimination/ partner choice or kin fidelity/ partner feedback fidelity mechanisms[76]. In case of kin discrimination/ partner choice, a cooperating kin/ partner is selectively chosen over a non-cooperating kin/ partner. In case of kin fidelity/ partner feedback fidelity, an interaction between the partners that cooperate creates a positive feedback and gets strengthened over time, and such partners are selected over a non-cooperating partner. But a community of microbes cooperating a common good can also be negatively affected by cheaters arising due to mutations or migration, which do not produce the common good but use it. In such

cases, the community might either reach extinction by exhaustion of the common good by the cheaters, leading to the 'tragedy of the commons' or it can be saved if cooperators are either spatially organized with each other or win the 'adaptive race' of fitness to changing environments[76–78]. Apart from this, microorganisms are known to help other microbes in the community even at their own expense, an interaction termed as altruism[79].

1.5.2 Negative interactions

When interactions between two organisms negatively affect one of them but do not affect the other, they are defined as amensalism[80]. But when two microbes involved in interactions are both harmed by each other, then they are classified as competition[81]. Cooperative interactions are usually thought to stabilize microbial communities, but interactions that are negative, such as a competition for resources, can also shape the microbial communities[82]. In certain cases, it has been observed that one of the microbe feeds on the other microbe, giving rise to the phenomena of predation[83]. It has been observed that certain bacteria can kill their siblings, lyse their cells, and feed on the nutrients released under starvation conditions like sporulation, a process termed as cannibalism[84]. In yet another predatory process called fratricide, competent cells have been shown to lyse their non-competent siblings for getting DNA and nutrients[85].

1.6 Types of metabolites exchanged among microbes

Interactions among microorganisms in a community are also shaped by a multitude of metabolites released and exchanged by them as mentioned earlier. Complex natural microbial communities associated with animals and plants are involved in exchange of nutrients such as carbon sources, amino acids, nucleobases, vitamins, and minerals[39,67]. Human gut microbial communities can degrade complex polysaccharides to simple sugars[12]. Exchange of amino acids among microbial mutants has been extensively utilized to understand the evolutionary capabilities of bacteria[5,36–38,71]. Exchange of purines such as adenine, hypoxanthine, and xanthine has also been observed among microbes and between microbes and their hosts[6,43,86]. Molecules ranging from single electrons to siderophores involved in mineral sequestration are also exchanged among co-occurring microbes[7,8]. Water-soluble vitamins of group B and intermediates in their biosynthesis pathways are also exchanged among microbial members in gut and aquatic ecosystems[68,87]. Of these vitamins, auxotrophy (inability to synthesize a nutrient) for vitamin B_1 (thiamin), is also

observed among microbes. Thiamin pyrophosphate/ diphosphate (TPP/ ThDP) is a cofactor involved in carrying out 'impossible' decarboxylation reactions for enzymes in central metabolism such as pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and acetolactate synthase, and also acts as a cofactor for other enzymes such as transketolase and phosphoketolase (Figure 1.3)[88].

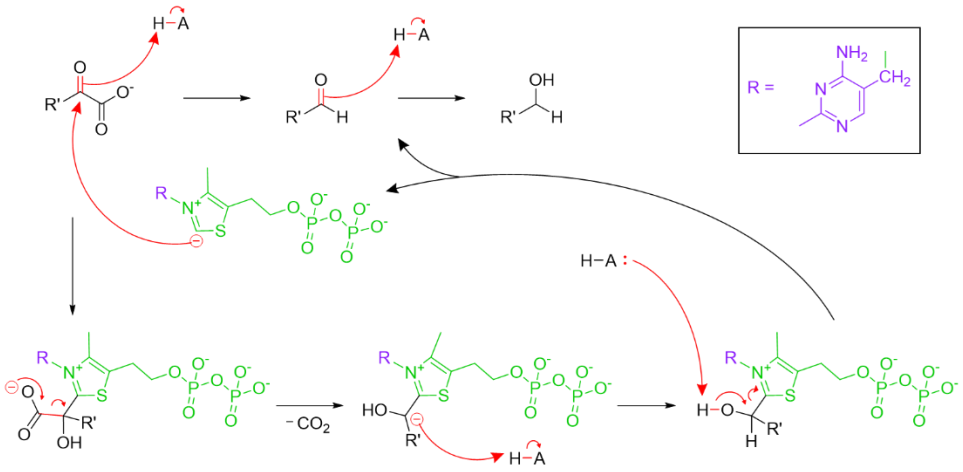

Figure 1.3 General mechanism of decarboxylation by thiamin. The ylide form of thiamin helps in difficult decarboxylation reactions.

1.7 Exchange of thiamin in microbial communities

1.7.1 Biosynthesis of thiamin in E. coli and intersection with purine biosynthesis pathway

Thiamin can be synthesized *de novo* by bacteria, plants, and fungi, but many other organisms such as humans and insects are unable to produce it, and rather acquire it from their diet and environment[89]. The *de novo* pathway for thiamin biosynthesis in *E. coli* forms thiamin by synthesizing and joining the two moieties of the vitamin in two separate branches of the pathway (Figure 1.4). The 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P) moiety is formed by the enzyme ThiC with a single-step rearrangement of its precursor 5'-Phosphoribosyl-5-aminoimidazole (AIR)[90]. AIR is synthesized by the enzyme PurM and is further committed to the biosynthesis of purines by PurK[91–93]. The 4-Methyl-5-(2-hydroxyethyl)thiazole (THZ) moiety is formed by the enzyme ThiG by combining 1-deoxy xylulose-5-phosphate (DXP), ThiS-thiocarboxylate, and tyrosine[94]. The sulfur in the THZ moiety is transferred through a cascade of

enzymes IscS, ThiI, ThiF and ThiS in the form of a thiocarboxylate group[95–97]. DXP is synthesized by Dxs, and the ThiH enzyme converts tyrosine to dehydroglycine[98,99]. The enzyme ThiD further phosphorylates HMP-P to HMP-PP, which is combined with THZ-P by the enzyme ThiE to synthesize thiamin monophosphate (ThMP)[100,101]. ThMP is further phosphorylated to thiamin pyrophosphate/ diphosphate (TPP/ ThDP) by the enzyme ThiL, the active form of the cofactor[102]. ThiK phosphorylates thiamin to ThMP; and ThiM phosphorylates THZ to THZ-P[102–104].

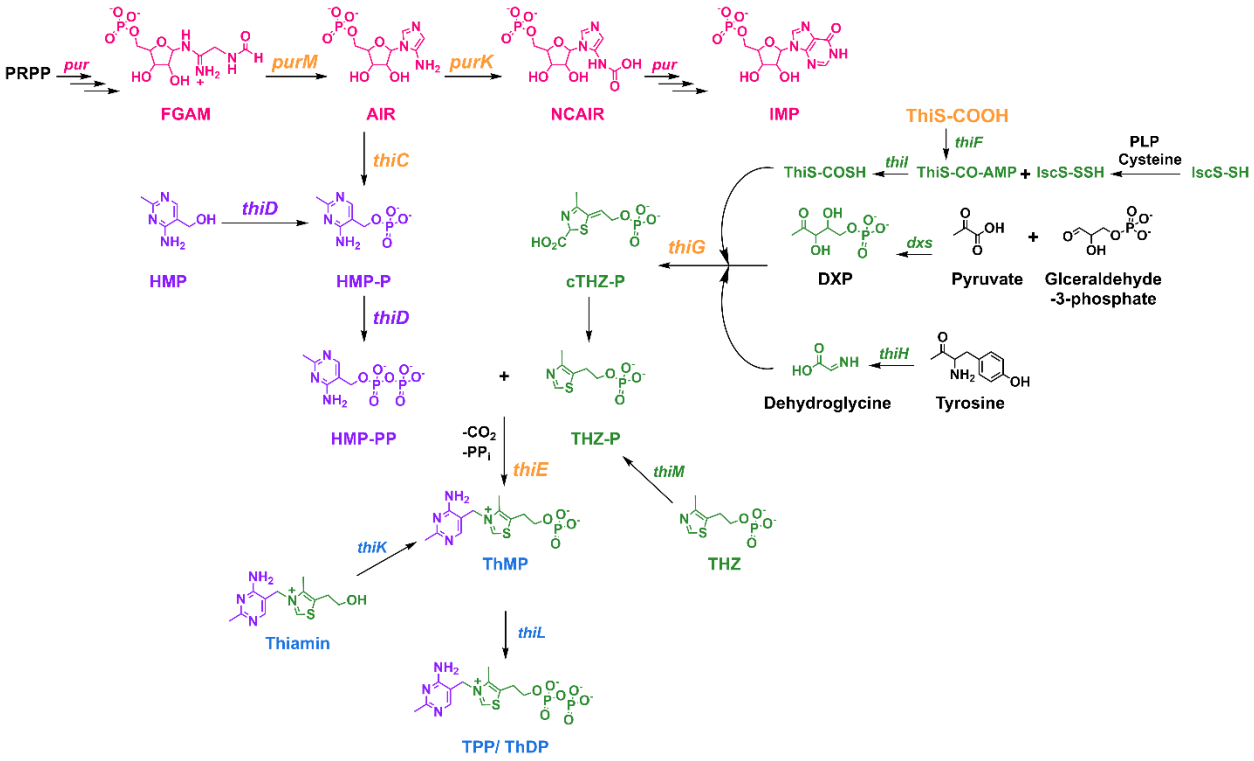

Figure 1.4 Thiamin and purine biosynthesis pathways in *E. coli* K-12 MG1655. Thiamin biosynthesis pathway in *E. coli* intersects the purine biosynthesis pathway at their common intermediate AIR. Biosynthesis of purine – pink, HMP – purple, THZ – green, and thiamin – blue. Mutants used in this study are denoted in golden colour. Abbreviations: FGAM, 2-(Formamido)-N1-(5'-phosphoribosyl)acetamidine; AIR, 5'-phosphoribosyl-5-aminoimidazole; NCAIR, 5-Carboxyamino-1-(5'-phosphoribosyl)imidazole; IMP, Inosine 5'-monophosphate; HMP, 4-amino-5-hydroxymethyl-2-methylpyrimidine; HMP-P, HMP phosphate; HMP-PP, HMP diphosphate; PLP, pyridoxal phosphate; DXP, 1-deoxyxylulose-5-phosphate; THZ, 4-methyl-5-

(2-hydroxyethyl)thiazole; THZ-P, THZ phosphate; cTHZ-P, THZ phosphate carboxylate tautomer; ThMP, thiamin monophosphate; TPP/ ThDP, thiamin pyrophosphate/ diphosphate.

1.7.2 Exchange and transporters of thiamin and its biosynthesis intermediates

Thiamin auxotrophy is prominent at the levels of both genus and family in human gut microbiota, indicating that it might be shared readily among them[68]. Thiamin is also known to undergo photodegradation to form its intermediate HMP which is more stable and shown to be present in a higher amount than thiamin in aquatic systems[105,106]. Auxotrophy for HMP in organisms such as *Pelagibacter ubique* growing in these waters is also predicted to help them with genome streamlining, whereby reduced genome size due to absence of genes like ThiC reduces the metabolic burden on them[106,107]. Algae such as *Emiliania huxleyi* and *Pavlova* sp. which contain all thiamin biosynthesis proteins except THIC are shown to have better growth on HMP and 4-amino-5-aminomethyl-2-methylpyrimidine (AmMP) than thiamin[108]. AmMP can be salvaged via the enzyme TENA_E present in these algae which converts it to HMP, which can be further used to synthesize thiamin. Both HMP and thiamin have been predicted to be prominently exchanged among microorganisms in human and insect gut and aquatic ecosystems, based on metagenomic data, but reports predicting THZ exchange are limited[4,68,109]. Apart from these, studies have shown exchange of thiamin among microorganisms, but reports showing exchange of HMP and THZ are scarce[110–112]. Organisms that export HMP in their surrounding medium have also been studied[106]. An analogue of THZ, carboxythiazole, has also been shown to be used preferentially over THZ, and thiamin biosynthesis pathway contains higher number of such non-canonical precursors among other vitamins[68,113]. A number of pathway variants of thiamin biosynthesis pathway have also been observed in human gut, analyzed using metagenomic data[68].

Theoretical and experimental studies show that certain thiamin auxotrophs do not take up thiamin as they lack its transporters. Instead, they salvage HMP and/ or THZ via their transporters, and subsequently phosphorylate and link them together to make thiamin for their survival[4,68,106,109,112]. Of these, presence of transporters for HMP and thiamin in organisms has been majorly reported in literature, whereas only putative roles have been predicted for some proteins in THZ transport (Figure 1.5) [4,114–118]. Also, primary ABC family transporters of thiamin such as

ThiT which help in uptake of thiamin are prevalently found in auxotrophs, whereas secondary transporters such as PnuT which facilitate its bidirectional transport are more prevalent in prototrophs[4,68].

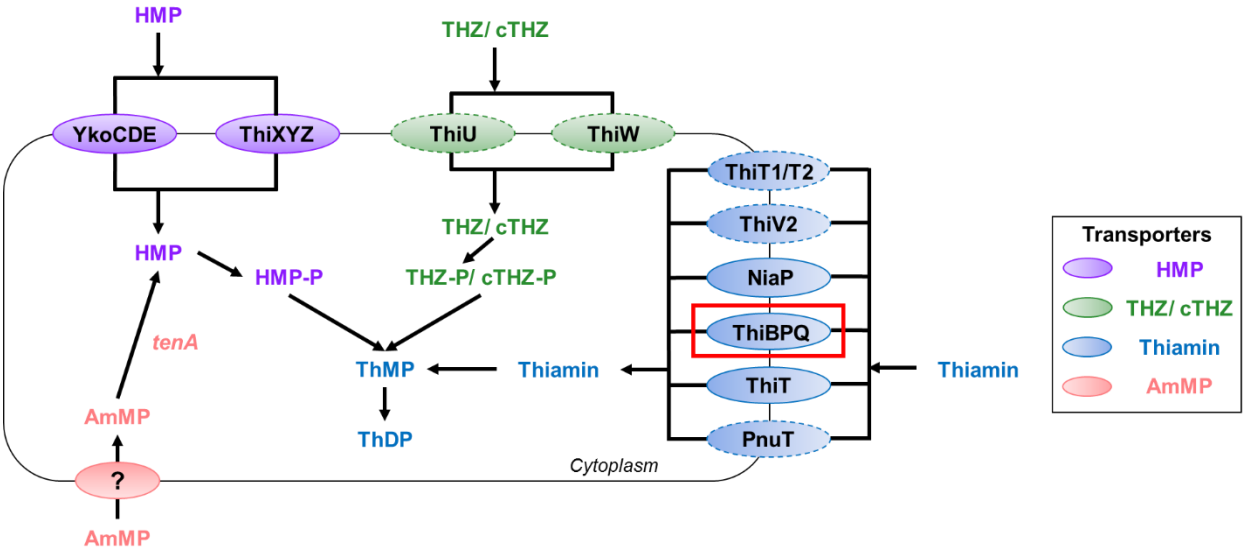

Figure 1.5 Transporters of thiamin, HMP and THZ. Thiamin and its biosynthesis intermediates HMP and THZ have various transporters reported, of which, ThiBPQ is the only transporter present in *E. coli*. Dashed lines represent putative transporters.

1.7.3 Analyzing and detecting exchange of thiamin

Taken together, the studies mentioned earlier point to exchange of thiamin and its biosynthesis intermediates between microorganisms. Thiamin biosynthesis pathway has two distinct intermediates synthesized in two separate branches of the pathway, making the mutational analysis easier. Apart from these, sensitive assays such as the thiochrome assay which converts thiamin to its fluorescent derivative and LC-MS/MS using standards of thiamin and its intermediates are available for detection of thiamin[119,120]. Also, of the transporters reported, only one thiamin transporter, ThiBPQ, is present in *E. coli*[89,118]. Thus, we decided to analyze the

exchange of thiamin and its biosynthesis intermediates HMP and THZ in synthetic co-cultures of *E. coli* str. K-12 substr. MG1655 thiamin biosynthesis mutants.

For this, we created the single gene knockout mutants of *E. coli* thiamin biosynthesis pathway genes, *ΔthiC*, *ΔthiE*, and *ΔthiG*, and analysed their physiology in Chapter 2. In Chapter 3, we studied the exchange of thiamin and its biosynthesis intermediates among pairwise co-cultures of these mutants. Here, we observed that thiamin and HMP are exchanged prominently in *E. coli* co-cultures, whereas exchange of THZ does not occur in them. We also quantified the ratio of mutants in these co-cultures, and observed that it varies in the absence of thiamine, indicative of a cross-talk, but does not vary when thiamin is available in plenty. Thiamin and purine biosynthesis pathways in *E. coli* share a common intermediate, AIR, and thus specific mutants of these pathways might also engage in a cross-talk which is dependent on exchange of particular intermediates and flux through one pathway might affect another. Thus, in Chapter 4, we analyzed the exchange of intermediates between purine and thiamin biosynthesis pathway mutants of *E. coli*. This analysis also pointed to the prevalent exchange of HMP in *E. coli* co-cultures, along with exchange of thiamin and inosine or some other purine intermediate, leading to survival of specific mutant pairs under varied conditions. We also visualized the spatial organization of thiamin biosynthesis mutants as a part of Chapter 5, where we observed co-localization of interacting mutants in the absence of thiamin, a phenomenon not observed in its absence. In Chapter 6, we discuss other physiological explorations that we performed in *E. coli*. In Chapter 7, we summarize our findings and enlist the questions posed by this thesis that yet remain unanswered.

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Chapter 2: Phenotypic analysis of thiamin biosynthesis pathway mutants of *Escherichia coli* K-12 MG1655

2.1 Thiamin biosynthesis pathway mutants of *E. coli* K-12 MG1655

Thiamin is a vitamin synthesized by bacteria, plants, and fungi, and its biosynthesis via *de novo* and salvage pathways in these organisms was discussed in Chapter 1, Figure 1.4. Thiamin biosynthesis pathway in *E. coli* involves the formation of HMP and THZ in two separate branches of the pathway, by ThiC and ThiG, respectively, and these two moieties are further joined together by ThiE[2]. *E. coli* str. K-12 substr. MG1655 is the variant of *E. coli* that is widely used for analyzing the physiology of this bacterium, and the neighbourhood of *thi*$^{-}$ genes in it is shown in Figure 2.1.

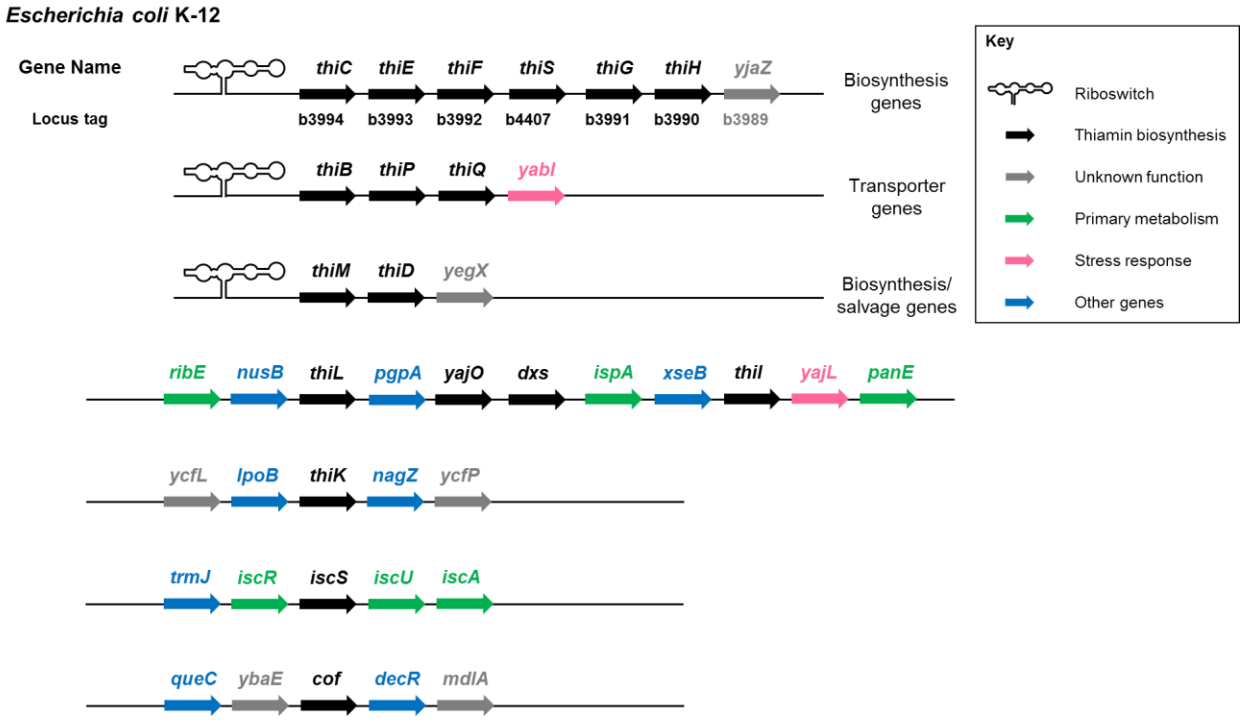

Figure 2.1 Thiamin biosynthesis genes in *E. coli* K-12 MG1655. Majority of thiamin metabolism genes in *E. coli* are arranged in the form of three operons regulated by ThDP-dependent riboswitches: (i) *thiCEFSGH*, coding for genes involved in *de novo* thiamin

biosynthesis, (ii) *thiBPQ*, coding for an ABC-type bidirectional thiamin transporter and (iii) *thiMD*, coding for kinase genes involved in both *de novo* and salvage pathways for thiamin biosynthesis. Sathe *et al*, J Bacteriol, 2022.

Based on the *de novo* biosynthesis pathway in *E. coli*, we hypothesized that the mutants in the HMP and THZ biosynthesis branches of the pathway would have complementary requirements for HMP and THZ for their growth. We further hypothesized that in a co-culture without exogenous thiamin supplementation in a minimal medium, these mutants might be able to exchange these nutrients with each other, leading to the growth of the co-culture. In this chapter, we describe the process by which the *E. coli* K-12 MG1655 single knockout mutants *ΔthiC*, *ΔthiE*, and *ΔthiG* of thiamin biosynthesis pathway (referred to as *thi*$^-$ mutants) were created. Further, we compared their growth in a rich medium to that of the wild type (WT) strain to check for any additional stress due to mutations.

We next analyzed whether the complementation of *thi*$^-$ mutants with plasmids containing genes corresponding to the mutations rescues their thiamin deficiency. As thiamin biosynthesis genes in *E. coli* are present in a single operon, checking whether the complementation allows for rescue of thiamin deficiency became essential (Figure 2.1)[3]. We also checked whether *thi*$^-$ mutants can be complemented with HMP, THZ, THZ-P, and thiamin, and the concentration at which the uptake occurs for these nutrients. Confirming the phenotypes of *thi*$^-$ mutants in both these ways will allow for their utilization for analyzing the exchange of thiamin and its biosynthesis intermediates.

We observed that all three *thi*$^-$ mutants do not have any growth disadvantage when compared to the WT strain. We also observed that these mutations can be complemented by supplementing them with either the plasmids carrying the respective genes or HMP, THZ, or thiamin. The *ΔthiC* strain survived with HMP or thiamin, the *ΔthiG* strain survived with THZ or thiamin, whereas the *ΔthiE* strain survived with only thiamin supplementation, as expected from their genotypes.

2.2 Materials and methods

2.2.1 Chemicals and reagents: All the chemicals, reagents and kits used were obtained either from TCI chemicals, HiMedia or Sigma unless otherwise specified. The enzymes used for generating knockouts, diagnostic PCRs, and cloning were obtained from TaKaRa.

2.2.2 Strains and plasmids: The *E. coli* K-12 BW25113 strain containing the plasmid pKD46, the *E. coli* K-12 BW25141 strain containing the plasmid pKD4, and the empty pProEx-Hta plasmid were a gift from Dr. Nishad Matange, IISER Pune.

2.2.3 Generating single gene knockouts in E. coli: The λ-red recombineering system was used for creating the single gene knockout mutants of *E. coli* K-12 MG1655, and steps for primer design, mutant generation, and verification were carried out as previously described[4,5].

2.2.3.1 Designing PCR Primers: Primers listed in table 2.1 were designed for generating gene knockouts and for their confirmation. The N-terminal deletion primers consisted of a 50-nt extension upstream of the gene, including the initiation codon of the same, followed by a 20-nt long stretch corresponding to the priming site 1 (P1) region of the plasmid pKD4. The C-terminal deletion primers consisted of 18-nt for the C terminal region of the gene, the stop codon, and 29-nt downstream of the same followed by a 20-nt long stretch corresponding to the priming site 2 (P2) region of the plasmid pKD4. The forward primers used for diagnostic PCRs to verify the gene knockouts consisted of about 20-25-nt long sequences, selected upstream of the gene, and the reverse primer used consisted of a sequence of 24-nt inside the kanamycin cassette on the vector pKD4.

2.2.3.2 Generation of PCR fragments: Deletion primers were used to amplify fragments of the kanamycin cassette from the vector pKD4 flanked with the regions corresponding to the gene to be deleted. The amplified PCR fragments were analyzed using 1% agarose gel electrophoresis, followed by either gel extraction or PCR purification.

2.2.3.3 Electroporation and mutant selection: *E. coli* K-12 MG1655 WT cells carrying pKD46 (a plasmid containing λ-red recombinase machinery, Amp^R, transformed by making chemically-competent cells) were made electro-competent for transformation. Briefly, cells were grown in [LB + Ampicillin (100 µg/ mL)] aerobically at 30°C, 180 rpm in an overnight primary culture and used to start a 30 mL secondary culture (5 mL culture in 6 X 50 mL falcons) with 3% inoculum in [LB + Ampicillin (100 µg/ mL)] at 30°C, 180 rpm, with 0.2% arabinose, 3 h. The culture was then centrifuged at 3000 rpm, 10 minutes, at 4°C and washed thrice with 10

mL of ice-cold Milli-Q, pooling cells with every wash. Finally, cells were resuspended in 300 µL of ice-cold Milli-Q and divided into 3 equal parts in electroporation cuvettes, pre-chilled on ice. PCR fragments (~100 ng) containing the kanamycin cassette with flanking regions corresponding to the gene to be deleted were added to each cuvette. Cells were electroporated at 2.5 kV followed by immediate addition of 1 mL LB. After recovery for 4 h at 37°C, 180 rpm, 500 µL of cells were plated onto LB plates with 25 µg/ mL kanamycin to select for Kan^R transformants and incubated at 37°C, 12 h. Growth at 37°C cures the cells of pKD46, as it contains a temperature-sensitive origin of replication[5,6]. Colonies thus obtained were re-streaked on LB plates with kanamycin – with or without ampicillin, and the plates were incubated at both 30°C and 37°C, 12 h to check if pKD46 has been cured.

2.2.3.4 *Diagnostic PCR for the verification of deletions:* The mutants generated were verified using colony PCR. Briefly, a colony each for a mutant was resuspended in 5 µL Milli-Q in a PCR tube, and a PCR reaction was conducted to check for the deletion. The control reactions were carried out using a colony from the wild type strain. The amplified PCR fragments were analyzed using 1% agarose gel electrophoresis and software Fiji, and were confirmed based on their sequencing results[7].

2.2.4 Growth conditions and media: *E. coli* K-12 MG1655 cells were grown either in LB or in M9 minimal media at 37°C, 180 rpm[8]. Whenever necessary, the media were supplemented with various components in small defined amounts as mentioned.

2.2.4.1 *Primary culture set-up (LB and P1 cultures):* *E. coli* K-12 MG1655 WT, *ΔthiC*, *ΔthiE*, and *ΔthiG* strains were grown in LB aerobically at 37°C, 180 rpm, for 6-8 hours. The cultures were centrifuged at 6500 rpm for 1 minute and the pellets were washed thrice with 1X M9 salts by re-suspending them using a vortex for each wash. This step was used to make sure that cells do not carry-over any residual nutrients from the LB medium. These cells were used to start P1 cultures (first subcultures in M9 medium) in [M9 + NH_4Cl + Glucose] medium, at a starting OD_{600} of 0.05, and were incubated aerobically at 37°C, 180 rpm, for 16-18 hours. Cells grown in P1 were centrifuged at 6500 rpm for 1 minute and the pellets were washed thrice with 1X M9 salts by re-suspending them using a vortex for each wash. This step was used to make sure that the cells do not carry-over any residual nutrients from the P1 stage.

2.2.4.2 *Secondary culture set-up (P2 cultures):* Cells washed after P1 were used to start their growth curves in P2 (second passage) in [M9 + NH_4Cl + Glucose] medium, at a starting OD_{600}

of 0.1, and were incubated at 37°C for 24-48 h, 180 rpm. Cultures with 2 mL or 4 mL volumes were set up in 15 mL or 25 mL test tubes in shaker incubators (Thermo Scientific or Scigenics). Cultures were also started as 200 µL cultures in a 96-well plate, at 200-300 rpm in Varioskan (Thermo Scientific), M200 Pro (Tecan), EnSight (Perkin-Elmer), or CLARIOstar.

2.2.4.3 *Mutant phenotype rescue:* The gene *thiE* from *E. coli* K-12 MG1655 was cloned in pProEx-Hta vector using primers listed in table 2.1 and restriction-free cloning method as previously described[9]. The empty pProEx-Hta vector and the *EcthiE*-pPRoEx-Hta vector were then chemically transformed into the *E. coli* K-12 MG1655 *ΔthiE::kan*^{R} strain for complementation analysis. Various pCA24N vectors were purified from the ASKA library (hosted in Hazra lab, IISER Pune) using the HiMedia Plasmid MiniPrep kit. *EccobT*-pCA24N vector was chemically transformed into *E. coli* K-12 MG1655 *ΔthiC::kan*^{R} and *ΔthiG::kan*^{R} strains for complementation analysis. *EcthiC*-pCA24N and *EcthiG*-pCA24N plasmids were chemically transformed into *E. coli* K-12 MG1655 *ΔthiC::kan*^{R} and *ΔthiG::kan*^{R} strains, respectively. Cultures of transformed and untransformed *ΔthiC::kan*^{R}, *ΔthiE::kan*^{R}, and *ΔthiG::kan*^{R} strains were analyzed in the P2 passage for complementation assays.

2.2.4.4 *Feeding studies:* Thiamin and THZ stock solutions used were prepared in distilled water as a solvent, whereas HMP stocks were made in 0.1% dimethyl sulfoxide (DMSO). These stocks were then filter-sterilized using 0.22 µM filters and stored at -20°C till further use. For regular use, kanamycin, thiamin and inosine stocks were prepared at 1000X concentrations of the final concentrations used in the cultures. For feeding studies, 10 µL of HMP, THZ, THZ-P and thiamin stocks (20X concentration of the final concentrations used in the cultures) were used for 200 µL cultures in the 96-well plate. The P2 monocultures and co-cultures were started with or without either HMP, THZ, THZ-P or thiamin at final concentrations of 1 pM, 100 pM, 1 nM, 100 nM and 1µM for the feeding studies. For HMP feeding, additionally, 0.1% DMSO was fed to the cultures as a negative control. For THZ-P feeding, the THZ-P reaction with *Ec*ThiM enzyme was diluted appropriately to get stocks of 20X concentration of the final concentrations used in the cultures. As the THZ-P reaction showed complete conversion of THZ to THZ-P, concentration of THZ-P in the reaction was considered to be the same as the initial THZ concentration (0.5 mM) utilized in the reaction.

Sr. No.	*Primer name/ purpose*	Primer Sequence (5' → 3')
1.	*thiC::kanR_for*	TCATCCGTCGTCTGACAAGCCACGTCCTTAACTTTTTGGAA TGAGCTATGGTGTAGGCTGGAGCTGCTTC
2.	*thiC::kanR_rev*	AGGTACAGGAGGAAAATCAGGCTGATACATCACGCTTCCT CCTTACGCAGCATATGAATATCCTCCTTAG
3.	*thiE::kanR_for*	TTCCGTGCCAGAGGCGGAGAAATCTACCTGCGTAAGGAGG AAGCGTGATGGTGTAGGCTGGAGCTGCTTC
4.	*thiE::kanR_rev*	CCCGTCCAGAGCGATATCGTCGAGCAGGATCATTCATCGC CAACTCCTGCCATATGAATATCCTCCTTAG
5.	*thiG::kanR_for*	GGATGGCGACCAGATCCTGCTTTTTCAGGTTATTGCAGGG GGTTGAAATGGTGTAGGCTGGAGCTGCTTC
6.	*thiG::kanR_rev*	CAGTTGTCGCCAGCGATCGCTGAAGGTTTTCATGCCGATG CCTCCAGAAACATATGAATATCCTCCTTAG
7.	*thiC::kanR_conf_for*	CGAAGGGAACAAGAGTTAATCTGC
8.	*thiE::kanR_conf_for*	ATTTTTGCTCCATGTGTGGG
9.	*thiG::kanR_conf_for*	CAAACTGTTCACGAACTACTGGAG
10.	*kanR_conf_rev*	TAATCAGCACCTGGCTGTCG
11.	*Ec_thiE_PCR1_for*	ATGTATCAGCCTGATTTTCC
12.	*Ec_thiE_PCR1_rev*	TCATTCATCGCCAACTCC
13.	*Ec_thiE_PCR2_for*	TTCAGGGCGCCATGGATCCGATGTATCAGCCTGATTTTCC
14.	*Ec_thiE*_PCR2_rev	TACCGCATGCCTCGAGACTGTCATTCATCGCCAACTCC
15.	*Ec_thiM*_PCR1_for	ATGCAAGTCGACCTG
16.	*Ec_thiM*_PCR1_rev	TCATGCCTGCACCTC

17.	*Ec_thiM*_PCR2_for	CCGCGCGGCAGCCATATGCAAGTCGACCTG
18.	*Ec_thiM*_PCR2_rev	ACGGAGCTCGAATTCGGATCCTCATGCCTGCACCTC

Table 2.1 Sequences of primers used in Chapter 2. Key: for = forward primer, rev = reverse primer, kan = kanamycin, conf = diagnostic PCR (confirmatory) primer.

2.2.5 Enzymatic synthesis of THZ-P using ThiM

2.2.5.1 Cloning, overexpression and purification of EcThiM: The thiazole kinase ThiM from *E. coli* K-12 MG1655 was cloned in pET28a vector using restriction-free cloning method as previously described[9]. The *Ec*ThiM-pET28a plasmid was further transformed into *E. coli* DH5-α cells, selected for kanamycin resistance and the clones were confirmed by sequencing using vector-specific T7 primers. The *Ec*ThiM-pET28a plasmid was further transformed into *E. coli* BL21(DE3) cells for overexpression of *Ec*ThiM. Overnight primary cultures of *E. coli* BL21(DE3) cells containing the *Ec*ThiM-pET28a plasmid were grown in LB with kanamycin (25 µg/ mL) at 37°C, 180 rpm. These cultures were transferred into large secondary cultures of LB with kanamycin (25 µg/ mL) medium at 1% v/v inoculum and were grown at 37°C, 180 rpm to an OD_{600} of 0.6. Protein overexpression was achieved with 0.1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG), following which cultures were further incubated at 37°C for 3 h, 180 rpm, and protein was purified as described earlier[10]. Cells were harvested from large cultures by centrifugation at 6500 rpm, 4°C and pellets were flash frozen in liquid nitrogen and stored at -80°C till further use. Cell pellets obtained were resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0) containing 300 mM NaCl, 10 mM $MgCl_2$, and 0.05 mM phenylmethylsulfonyl fluoride and were lysed by sonication. The cell lysate was loaded onto a 5 mL pre-packed Ni-NTA column (Bio-Rad) pre-incubated with 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl, and 10 mM imidazole. The column was then washed with 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl, and 150 mM imidazole, and the protein was eluted in 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl, and 250 mM imidazole. Eluted protein fractions were separated on sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) to check for the presence of *Ec*ThiM. The purified protein was buffer-exchanged in 50

mM Tris-HCl, pH 8.0, containing 100 mM NaCl using Econo-Pac 10DG pre-packed desalting columns (Bio-Rad) and stored with 15% v/v glycerol at -80°C.

2.2.5.2 *In-vitro synthesis of thiazole phosphate (THZ-P) and detection using High Performance Liquid Chromatography (HPLC):* The purified protein *Ec*ThiM was used for the in vitro synthesis of THZ-P from THZ. Briefly, 0.5 mM of THZ and 5 mM of ATP, were incubated with 1 µM of *Ec*ThiM for 2 h in 100 mM Tris-HCl, pH 8.0, at 37°C. Reactions were quenched using 100 µL of 2M HCl per 200 µL reaction and analyzed using HPLC for the detection of THZ-P using a slight modification of the method previously described[10]. Briefly, the solvents used were: A – Milli-Q, B – Methanol, and C – 10 mM Ammonium acetate, pH 6.5. The gradient used was 0 min – 100% C, 5 min – 100% C, 6 min – 5% A, 5% B, 90% C, 16 min – 30% A, 30% B, 40% C, 21 min – 30% A, 30% B, 40% C, 25 min – 100% C. The flow rate was 1 mL/ min, with 100 µL injection volume. Both THZ and THZ-P were detected using absorbance at 251 nm.

2.3 Research design, Results, and Discussion

2.3.1 Generating single knockout mutant strains of thiamin biosynthesis pathway in E. coli K-12 MG1655

E. coli str. K-12 substr. MG1655 *ΔthiC::kan*^{R}, *ΔthiE::kan*^{R}, and *ΔthiG::kan*^{R} mutants were created using the protocol mentioned earlier (Table 2.1 – primers 1-6). Diagnostic PCRs were carried out to verify gene deletions and simultaneous insertion of the kanamycin cassette using specific primers for each mutant. The WT strain used as a negative control for colony PCR showed no product as it lacks the kanamycin cassette for the reverse primer to bind, but *thi*$^-$ mutants showed products of expected lengths at around 1200 bp (Figure 2.2, Table 2.1 – primers 7-10).

2.3.2 Growth phenotypes of thiamin biosynthesis mutants in LB

LB Miller broth was used to analyze growth phenotypes of *E. coli* K-12 MG1655 WT and *thi*$^-$ mutant strains. Growth rates of WT, *ΔthiC*, *ΔthiE*, and *ΔthiG* strains were 1.653 ± 0.095, 1.567 ± 0.197, 1.785 ± 0.122, and 1.368 ± 0.101 gen h^{-1}, respectively in LB, which were comparable (Figures 2.3A and 2.3D). This showed that these three *thi*$^-$ mutant strains grew to the same extent

as that of the WT strain in a rich medium, proving that they do not have any growth disadvantage as compared to the WT strain.

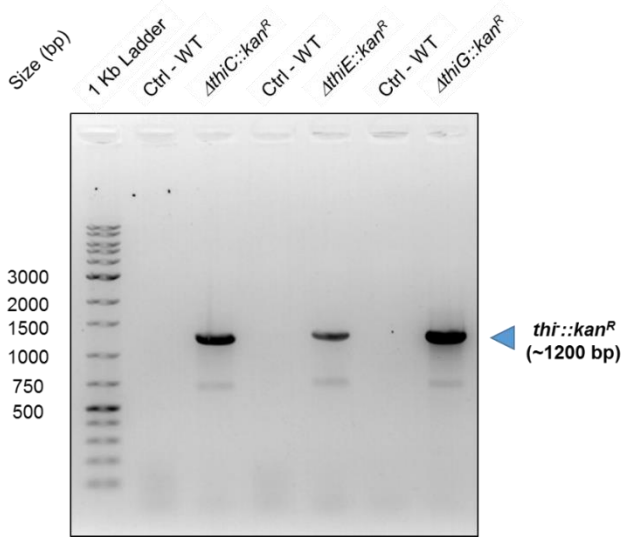

Figure 2.2 Diagnostic PCR for verifying gene deletions. Control reactions carried out using the WT strain do not show amplification, whereas those with mutants show deletion-specific products.

2.3.3 Growth phenotypes of thiamin biosynthesis mutants in M9 minimal medium

We next analyzed growth phenotypes of *thi*$^-$ mutants in M9-glucose minimal medium. In the first passage from LB to M9 (referred to as P1), we observed that all *thi*$^-$ mutants are able to survive equally well as the WT strain without thiamin supplementation (Figure 2.3B). This might be due to a carry-over of the nutrients from LB inside the cells. Such phenomenon has been observed before for other single knockout mutants of metabolic pathways in *E. coli*[5,11]. We hypothesize that as thiamin is a vitamin that can be regenerated and reused as a cofactor by the thiamin-utilizing enzymes, it is required only in catalytic amounts[12]. Thus, single cultures of *thi*$^-$ mutants can survive in the P1 passage of thiamin-deficient M9. Thus, we further cultured *thi*$^-$ mutants in a second passage of M9 (referred to as P2), where we observed that their growth occurred only at a basal level in comparison to the WT strain (Figure 2.3C). A third passage of mutants in M9 (referred to as P3) further reduced their growth to background levels, which would

have left very few cells for thiamin quantitation experiments (Figure 2.3C). Hence, we continued using the P2 passage for analyzing all further cultures and co-cultures.

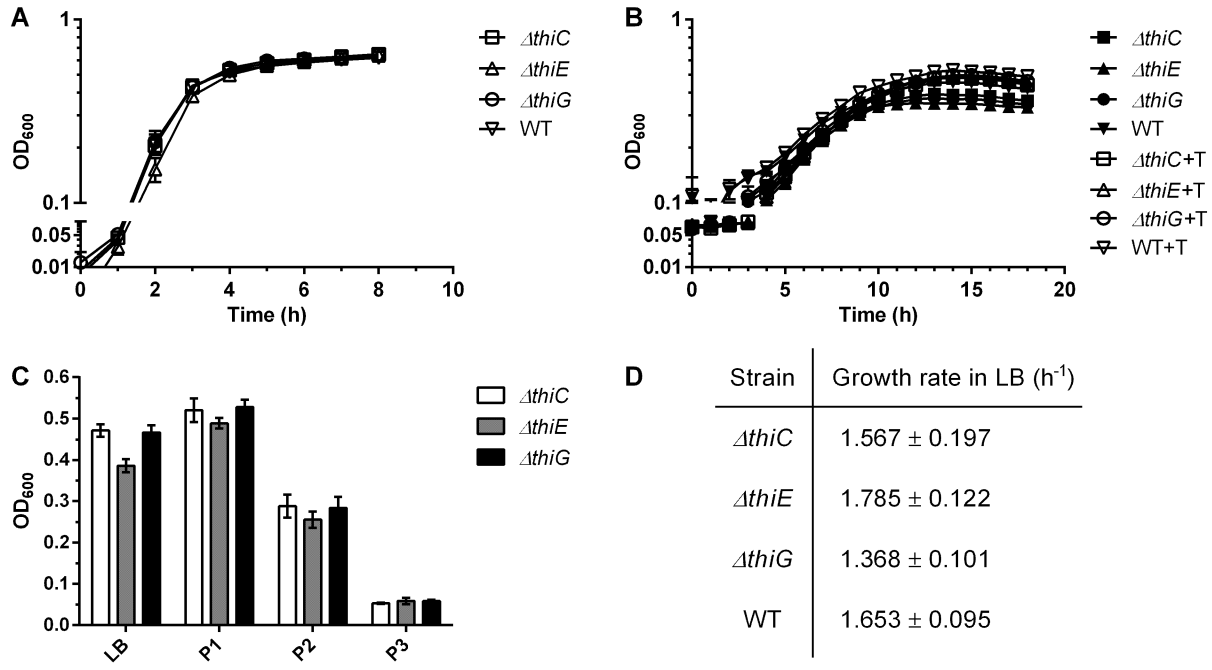

Figure 2.3 Growth phenotypes of *E. coli* thiamin biosynthesis mutants in LB and M9 media. Means ± standard errors of the means from three independent experiments are plotted. Growth phenotypes of *thi*$^-$ mutants in (A) LB and (B) P1. (C) Growth phenotypes of *thi*$^-$ mutants in P1, P2, and P3 at the end of 18h, 24 h, and 24 h, respectively, started at OD_{600} of 0.05, 0.1, and 0.1, respectively. (D) Growth rates of *thi*$^-$ mutants in LB. Sathe *et al*, J Bacteriol, 2022.

2.3.4 Complementation of thi$^-$ mutants for rescuing thiamin deficiency

The *E. coli* K-12 MG1655 *ΔthiC::kan^R*, *ΔthiE::kan^R*, and *ΔthiG::kan^R* mutants were checked for rescuing the thiamin deficiency caused due to mutations by using plasmids carrying the respective knocked-out genes. The WT strain was used as a positive control for growth. The

E. coli K-12 MG1655 *ΔthiC::kan*^{R} strain carrying the *EcthiC*-pCA24N vector, the *ΔthiG::kan*^{R} strain carrying the *EcthiG*-pCA24N vector, and the *ΔthiE::kan*^{R} strain carrying the *EcthiE*-pProEx-Hta vector grew well in the P2 passage even in the absence of any externally supplemented thiamin (Figure 2.4). On the other hand, strains carrying control vectors *EccobT*-pCA24N and empty pProEx-Hta did not grow well in the absence of external thiamin supply. This shows that the growth of thiamin biosynthesis mutants of *E. coli* K-12 MG1655 can be rescued by complementing the respective knocked-out genes on a vector in the absence of externally supplemented thiamin in M9-glucose minimal medium as observed earlier[13–15]. This experiment confirmed that *thi*$^-$ mutants exhibit the same phenotypes as expected from their genotypes, and thus can be utilized to specifically determine the exchange of thiamin and its biosynthesis intermediates.

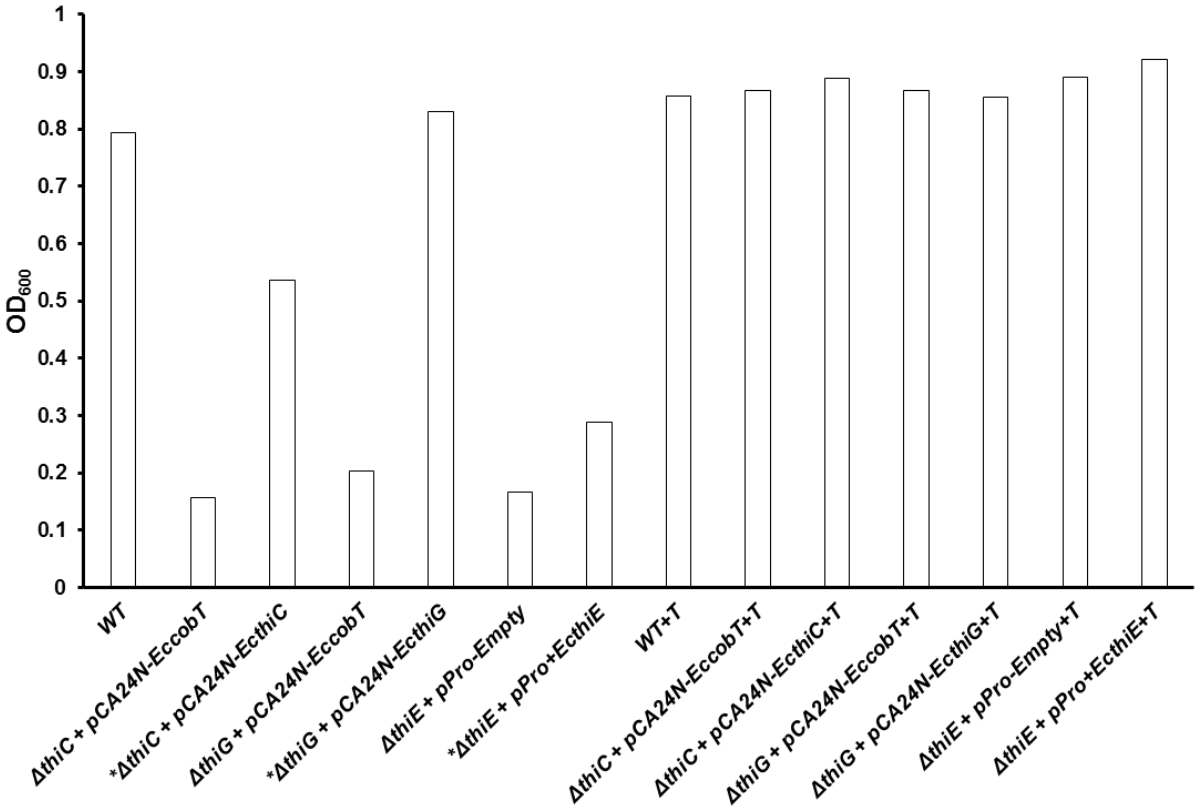

Figure 2.4 Phenotype rescue of *thi*$^-$ mutants by complementation with thiamin genes. Growth of *E. coli* thiamin biosynthesis mutants is restored in P1 after 18 h of growth when complemented

with a plasmid carrying the missing genes (indicated by *). For the wild-type and thiamin-supplemented controls, results of a single experiment are plotted; whereas for the rest, average values from two independent experiments are plotted. + T = with thiamin.

2.3.5 In vitro synthesis of THZ-P and detection using HPLC

The ThiM protein from *E. coli* was purified using Ni-NTA affinity chromatography, and the protein concentration was estimated to be 3 mg/ mL using absorbance at 280 nm. The SDS-PAGE gel showed an overexpressed band at 30.3 kDa eluting with 250 mM of imidazole washes, confirming that the protein purified is indeed *Ec*ThiM (Figure 2.5A). THZ-P synthesized in the enzymatic reaction of *Ec*ThiM was detected using absorbance at 251 nm with the help of HPLC. THZ (peak at 21.5 min) showed complete conversion to THZ-P (9.7 min) at the end of the 2 h reaction with ThiM (Figure 2.5B).

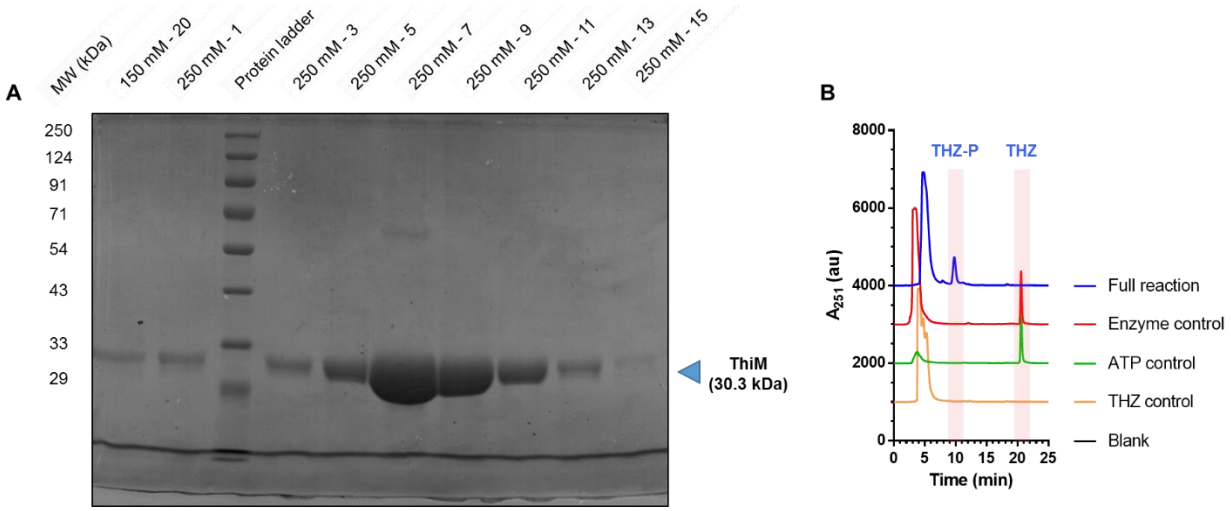

Figure 2.5 *In vitro* synthesis of THZ-P. (A) Protein purification gel for *Ec*ThiM. Numbers on top of wells represent imidazole concentration and the protein fraction used for loading, respectively. A band at 30.3 kDa shows purified *Ec*ThiM. (B) HPLC chromatogram for analyzing THZ-P synthesis by *Ec*ThiM, showing peaks for THZ and THZ-P at 21.5 min and 9.7 min, respectively.

2.3.6 Feeding HMP, THZ, and thiamin to thi⁻ mutants

Next, we fed single cultures of *thi*⁻ mutants with HMP, THZ, and thiamin in the P2 passage. We observed that all three thiamin biosynthesis mutants – *ΔthiC*, *ΔthiE*, and *ΔthiG* survive in the presence of thiamin, in a concentration-dependent manner, and the WT strain survives well even in the absence of thiamin (Figure 2.6). The *ΔthiC* strain survives with HMP and thiamin, but not with THZ (Figures 2.6, 2.7A, and 2.7D). Similarly, the *ΔthiG* strain survives with THZ and thiamin, but not with HMP (Figures 2.6, 2.7C, and 2.7F). But the *ΔthiE* strain survives only with thiamin supplementation, and not with HMP and THZ (Figures 2.6, 2.7B, and 2.7E).

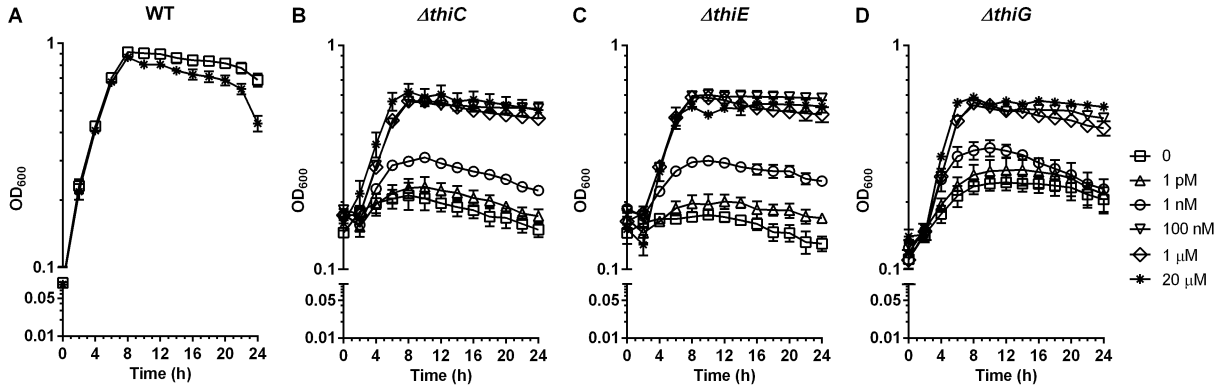

Figure 2.6 Supplementation of thiamin to thiamin mutants in P2 passage. Growth phenotypes of (A) WT, (B) *ΔthiC*, (C) *ΔthiE*, and (D) *ΔthiG* strains upon thiamin supplementation. Symbols represent following concentrations of thiamin: □ = Nothing added, Δ = 1 pM, ○ = 1 nM, ∇ = 100 nM, ◊ = 1 μM, * = 20 μM. Means ± standard errors of means from three independent experiments are plotted. Sathe *et al*, J Bacteriol, 2022.

All three strains show a concentration-dependent increase in their survival with HMP, THZ or thiamin. All three mutants show low growth with thiamin concentrations of up to 1 nM, but achieved an OD_{600} of ~0.6 with 100 nM, 1 μM and 20 μM thiamin. Concentrations of HMP at 100

nM or above facilitate the growth of the *ΔthiC* strain, whereas the response of the *ΔthiG* strain to THZ is more gradual and it starts to grow with THZ concentrations of 1 nM or above. This means that the phenotypes of *thi*$^-$ mutants correlate well with their genotypes as expected. This also means that *E. coli* has differential ability to uptake the two major precursors of thiamin, HMP and THZ.

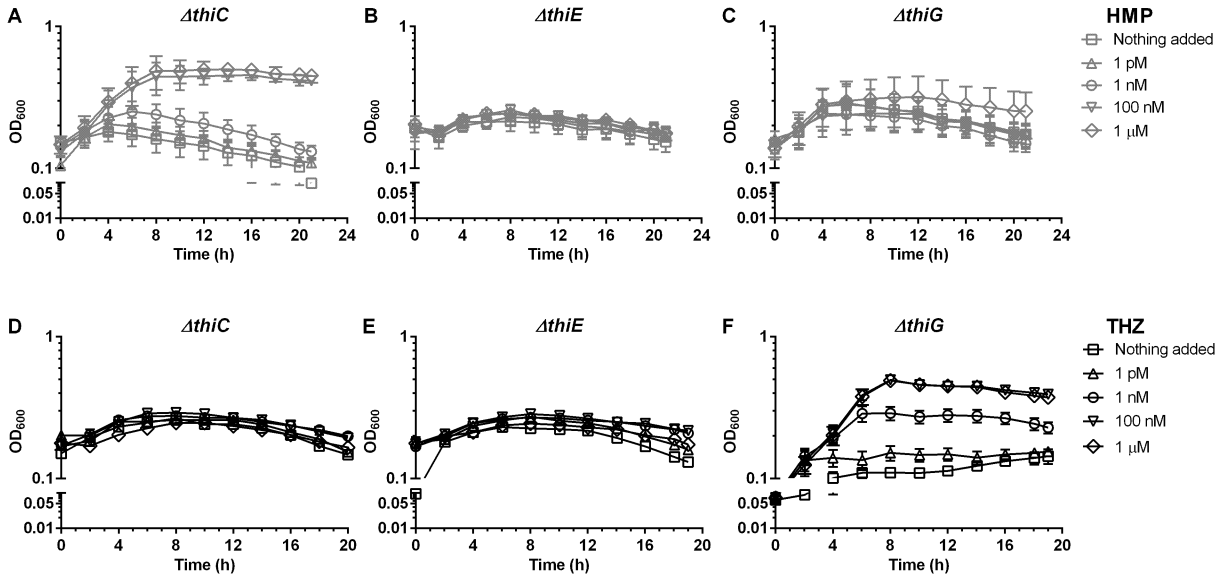

Figure 2.7 HMP and THZ supplementation to thiamin mutants of *E. coli*. Growth phenotypes of (A) *ΔthiC*, (B) *ΔthiE*, and (C) *ΔthiG* strains on HMP supplementation. Growth phenotypes of (D) *ΔthiC*, (E) *ΔthiE*, and (F) *ΔthiG* strains on THZ supplementation. Empty grey and black symbols represent HMP and THZ supplementation, respectively. Symbols represent the following concentrations: □ = Nothing added, Δ = 1 pM, ○ = 1 nM, ∇ = 100 nM, ◊ = 1 μM. Means ± standard errors of means from three independent experiments are plotted. Sathe *et al*, J Bacteriol, 2022.

2.3.7 Analyzing the uptake of THZ-P by E. coli

We next fed THZ-P to the *ΔthiG* strain to check if it is able to utilize THZ-P and survive in the P2 passage. As THZ is synthesized *de novo* in cells in the form of THZ-P, we hypothesized

that the presence of a polar group such as phosphate would hinder the uptake of THZ-P through the non-polar cell membrane. To our surprise, we observed that the *ΔthiG* strain survived in the presence of not only THZ, but also THZ-P at concentrations of 100 nM (Figures 2.7F and 2.8A).

To analyze whether THZ-P degrades in the medium to THZ which is then salvaged by the *ΔthiG* strain, we analyzed the control M9-Glucose medium supplemented with THZ-P and incubated at 37°C for different intervals of time (Figure 2.8B). But we could not detect peaks for either THZ or THZ-P even at 0 min, which might be due to either very low quantities of THZ and THZ-P being fed to the cultures that are out of the detection limit of HPLC-UV analysis or their degradation. Thus, it is possible that either the THZ or the THZ-P might supplement the growth of *E. coli ΔthiG* strain.

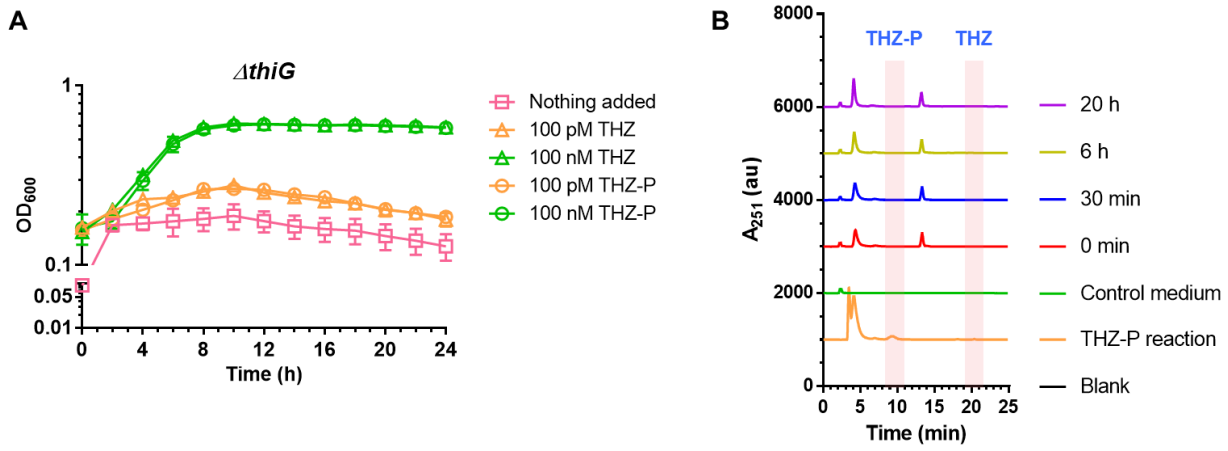

Figure 2.8 Feeding THZ-P to *E. coli*. (A) THZ-P supplementation to the *ΔthiG* strain. (B) THZ-P and THZ detection from control medium.

2.4 Summary

We created and confirmed three single gene knockout mutants of *E. coli* K-12 MG1655 – *ΔthiC::kan*^{R}, *ΔthiE::kan*^{R}, *ΔthiG::kan*^{R} and analyzed their growth phenotypes in M9 minimal medium under various conditions. We observed that these mutants do not exhibit a phenotypic defect in growth over the WT strain of *E. coli* K-12 MG1655 in a nutrient-rich medium, and hence

can be utilized for further experiments. We also observed that one passage in minimal medium is essential for *thi*$^-$ mutants for nutrient starvation, such that thiamin carry-over can be avoided.

Based on our feeding assays, we observed that all three *thi*$^-$ mutants have a requirement for thiamin, which acts as a growth-limiting nutrient in the P2 passage in M9 minimal medium. In order to ensure that thiamin is not limiting the growth of *thi*$^-$ mutants in the positive controls, we utilized 20 µM thiamin for all our future experiments. We also observed that *thi*$^-$ mutants have specific and complementary requirements for different thiamin biosynthesis pathway intermediates. The *ΔthiC* strain survives in M9 medium when supplemented with either HMP or thiamin. This is because it cannot synthesize HMP, but it can combine the HMP obtained from its environment with the THZ it synthesizes, form thiamin, and grow. Similarly, the *ΔthiG* strain cannot synthesize THZ, but survives in M9 medium with either THZ or thiamin supplementation. On the other hand, the *ΔthiE* strain can synthesize both the thiamin biosynthesis pathway intermediate HMP and THZ, but is unable to combine them to synthesize thiamin and hence it has a strict requirement for thiamin that it needs to uptake from its environment. Thus, owing to the complementarity of nutrient requirements, these three mutants can be utilized for analyzing the exchange of specific metabolites from the thiamin biosynthesis pathway.

We also analyzed the differential uptake of THZ and THZ-P by the *ΔthiG* strain, but the results were inconclusive when supplemented with THZ-P as it may form THZ in the medium. This experiment illustrates the difficulty of analyzing the exchange of phosphorylated analogues in the presence of buffers and media, which might degrade under these conditions, lowering their amount below the detection limits of assays. Methods with better detection limits and assays with suitable conditions are thus required for a detailed analysis involving exchanges of such molecules.

2.5 References

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Chapter 3: Analyzing the cross-talk among thiamin biosynthesis mutants of *Escherichia coli* K-12 MG1655

3.1 Possibilities for exchange of thiamin and its biosynthesis intermediates among thiamin biosynthesis pathway mutants of *E. coli* K-12 MG1655

E. coli contains the entire biosynthesis pathway for synthesizing HMP, THZ and thiamin *de novo* (Chapter 1, Figure 1.4)[1]. In *E. coli*, the HMP moiety is synthesized by the enzyme ThiC and the THZ moiety is synthesized by the enzyme ThiG, which are later joined to form thiamin by the enzyme ThiE (Chapter 1, Figure 1.4)[1]. As the *ΔthiC*, *ΔthiE*, and *ΔthiG* mutants of *E. coli* K-12 MG1655 have specific and complementary requirements for each of the three intermediates HMP, THZ and thiamin, we hypothesized that they might be able to exchange thiamin or the intermediates in co-cultures without thiamin supplementation (Chapter 2, Figures 2.6 and 2.7). Thus, we analyzed the growth of *thi*$^-$ mutants in three pairwise co-cultures - *ΔthiC-ΔthiE* (*CE*) co-culture, *ΔthiE-ΔthiG* (*EG*) co-culture, and *ΔthiC-ΔthiG* (*CG*) co-culture - in M9 minimal medium in the absence of thiamin in the P2 passage.

We hypothesize that the *CE* co-culture might be able to survive only if the *ΔthiE* strain supplements the *ΔthiC* strain with HMP, followed by the supplementation of thiamin from the *ΔthiC* strain to the *ΔthiE* strain owing to *ΔthiE* strain's stricter requirement for thiamin (Figure 3.1A). Similarly, the *EG* co-culture can survive only if the *ΔthiE* strain supplements the *ΔthiG* strain with THZ, followed by the supplementation of thiamin from the *ΔthiG* strain to the *ΔthiE* strain (Figure 3.1B). The *CG* co-culture, on the other hand, might be able to survive in thiamin-deficient M9 medium using three distinct possibilities: (i) the *ΔthiC* strain supplements the *ΔthiG* strain with THZ which supplements it with HMP in return, allowing both strains to synthesize thiamin on their own, (ii) the *ΔthiC* strain supplements the *ΔthiG* strain with THZ, which can now synthesize thiamin and provide it back to the *ΔthiC* strain, (iii) the *ΔthiG* strain supplements the *ΔthiC* strain with HMP, and the *ΔthiC* strain which can synthesize thiamin using the supplemented HMP provides thiamin back to the *ΔthiG* strain (Figure 3.1C).

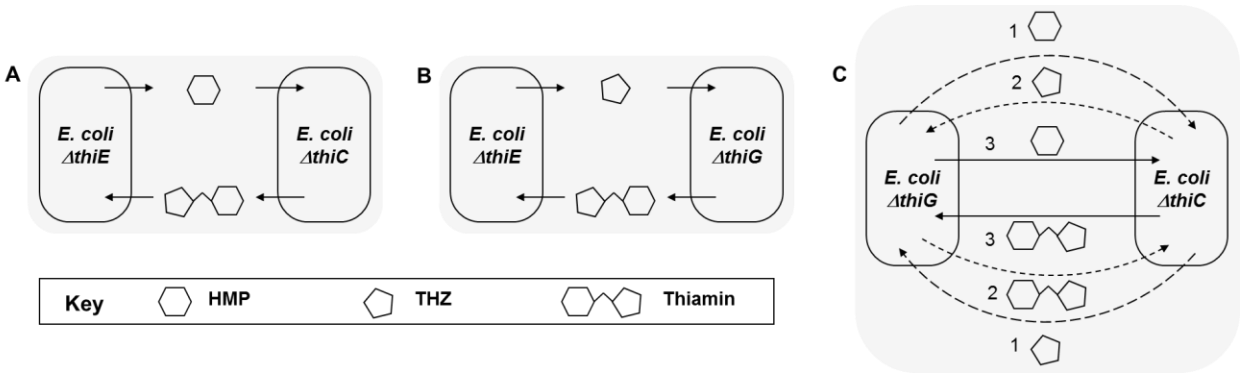

Figure 3.1 Models depicting possibilities of intermediate exchanges in pairwise co-cultures of thiamin mutants of *E. coli*. We hypothesize that (A) the *CE* co-culture might survive due to exchange of HMP and thiamin, (B) the *EG* co-culture might survive due to exchange of THZ and thiamin, and (C) the *CG* co-culture might survive if either (1) HMP and THZ are exchanged, or (2) THZ and thiamin are exchanged, or (3) HMP and thiamin are exchanged. Hexagon – HMP, pentagon – THZ, hexagon linked to pentagon – thiamin. Sathe *et al*, J Bacteriol, 2022.

To validate the exchanges of intermediates put forth by these hypotheses, we analyzed the growth of these three co-cultures in the presence and absence of thiamin in M9 medium. We further analyzed the amount of thiamin present in the co-cultures using a biochemical assay which converts thiamin and its phosphorylated derivatives to their fluorescent analogues, allowing for better detection of smaller amounts of thiamin from the co-cultures. We also checked whether the composition of the mutants in the co-cultures varies based on the availability of thiamin.

We observed that *CE* and *CG* co-cultures of *E. coli* survived in M9 media without external thiamin supplementation, whereas the *EG* co-culture did not grow. We further inferred that the growth of these co-cultures occurred due to exchange of HMP and thiamin between the strains, whereas the exchange of THZ seemed to be restricted, and could detect thiamin from their lysates. We also quantified the ratio of mutants in these co-cultures in the absence of exogenous thiamin and observed that their ratio changed from the value at start, indicative of a cross-talk among them. We also observed that their ratio did not vary when supplemented with thiamin, as they did not interact with each other.

3.2 Materials and methods

3.2.1 Chemicals and reagents: Thiamin, thiamin monophosphate, thiamin pyrophosphate and 4-methyl-5-hydroxyethyl thiazole were obtained from Sigma-Aldrich and 4-hydroxymethyl-2-methyl pyrimidine was obtained from TCI Chemicals. All other chemicals and reagents were obtained either from Sigma-Aldrich, HiMedia or TCI chemicals unless otherwise specified.

3.2.2 Strains and plasmids: The *E. coli* K-12 MG1655 strains *ΔthiC*, *ΔthiE*, and *ΔthiG* were constructed in this study (Chapter 2, Figure 2.2). The *E. coli* DH5α strain containing the plasmid pCP20 was a gift from Dr. Manjula Reddy, CCMB, Hyderabad. The *E. coli* KL-16 strain harboring the *GFPmut2-kan^R* cassette was a gift from Dr. Deepa Agashe, NCBS, Bangalore[2]. For generating strains marked with GFP, we flipped out the *kan^R* cassette from *thi$^-$* strains of *E. coli* K-12 MG1655 using a protocol previously described[3,4]. We then cloned and inserted the *GFPmut2-kan^R* cassette into them, after *aidB* gene, in the reverse orientation with respect to the *aidB* gene[2]. This gave us following strains – *ΔthiC aidB1633::GFPmut2-kan^R* (*ΔthiC**), *ΔthiE aidB1633::GFPmut2-kan^R* (*ΔthiE**) and *ΔthiG aidB1633::GFPmut2-kan^R* (*ΔthiG**). The *GFPmut2-kan^R* cassette insertions were carried out using following two strategies – recombination using the λ Red Recombineering system[4,5] and transduction using the P1 phage[6]. Diagnostic PCRs as described in Chapter 2, Materials and Methods, Section 2.2.3.4 and primers listed in Table 3.1 were used for verification of GFP-tagged mutants.

3.2.3 Growth conditions and media: *E. coli* K-12 MG1655 cells were grown either in LB or in M9 minimal media at 37°C, 180 rpm[7]. Whenever necessary, the medium was supplemented with various components in small defined amounts as mentioned.

3.2.3.1 *Primary culture set-up (LB and P1 cultures):* LB and P1 cultures of all strains were set up in the same manner as previously described (Chapter 2, Materials and Methods, Section 2.2.4.1).

3.2.3.2 *Secondary culture set-up (P2 cultures):* P2 cultures and co-cultures were set up following the same washing protocol as previously mentioned (Chapter 2, Materials and Methods, Section 2.2.4.2). Following this, for monocultures, the individual *thi$^-$* mutants were inoculated in [M9 + NH_4Cl + Glucose] medium with or without thiamin (50 µM), at a starting OD_{600} of 0.1, and were incubated either aerobically at 37°C, 180 rpm, for 24-96 hours. These co-cultures were started at either 1:9, 1:1 or 9:1 ratio of *thi$^-$* mutants, with individual cultures inoculated such that the total OD_{600} of co-cultures was 0.1 at the start. For example, for a *ΔthiC-ΔthiE* co-

culture started at 1:9 ratio of *ΔthiC*:*ΔthiE* strains with a starting OD_{600} of 0.1, the *ΔthiC* strain was inoculated at a starting OD_{600} of 0.01 and the *ΔthiE* strain was inoculated at a starting OD_{600} of 0.09, thus adding up to the 0.1 starting OD_{600} value.

3.2.3.3 Re-inoculation in passage P3: Cells from cultures in passage P2 were harvested at the end of 24 h, washed thrice with 1X M9 salts, inoculated at a starting OD_{600} of 0.1 in 2 mL fresh M9, and incubated at 37°C, 180 rpm, 24 h.

3.2.3.4 Experiments with alternate carbon sources: For experiments in which the carbon source was changed, 44.4 mM of Na-Pyruvate or 33.3 mM of Na-Succinate was used in place of 22.2 mM glucose for P2 cultures, all other conditions remained unaltered.

3.2.4 Thiochrome assay for detection of thiamin from cell lysates: *E. coli* K-12 MG1655 cells from 2 mL monocultures and co-cultures grown in the P2 passage under different conditions were centrifuged at 6500 rpm, 2 min, room temperature (RT) and cell pellets and spent media were then separated and stored at -20°C until further use. The cells were washed once with 1X PBS and re-suspended in 125 µL of 1X PBS, following which, they were lysed using a probe sonicator, 1 sec On and 3 sec Off cycles, total 1 min of On time, at 60 % amplitude, on ice to prevent the degradation of TMP and TPP. The cell lysate was separated from the debris by centrifuging the lysates at 14500 rpm, 30 min, 4°C and 100 µL of clarified lysates and spent media were then used for the thiochrome assay as described previously[8].

3.2.4.1 High Performance Liquid Chromatography (HPLC): Thiochrome and its phosphorylated analogues formed during the assay were detected using a High Performance Liquid Chromatography system coupled with a fluorescent detector (HPLC-FLD) from Agilent (1260 Infinity II) on a C-18 column (Phenomenex, Gemini, 5 µm, NX-C18, 110 Å, 250 × 4.6 mm) using reverse-phase chromatography with the following solvent system – MilliQ in line A, methanol in line B and 10 mM of ammonium acetate (CH_3COONa), pH 6.5 in Line C as described earlier[8]. The flow rate was maintained at 0.5 mL/ min with 100 µL of injection volume. Standard curves were generated using thiamin, TMP and TPP standards in the concentration range from 100 nM – 500 nM, treated with thiochrome assay. The LC method used was - 0 min: 100% A; 4 min: 90% A, 10% B; 20 min: 15% A, 25% B, 60% C; 24 min: 15% A, 25% B, 60% C; 30 min: 100% A; 44 min: 100% A, with detection of thiochrome at 370 nm, spectra were collected in the range of 190 nm - 600 nm using 5 nm bandwidth. TMP and TPP standards treated with thiochrome were kept in ice except for 1 min of assay treatment

and were injected immediately after the quenching step in the assay to avoid further heat-mediated degradation. Samples treated with thiochrome assay were centrifuged at 14500 rpm, 20 min before injection to precipitate the protein debris.

3.2.4.2 *Liquid Chromatography coupled tandem Mass Spectroscopy (LC-MS/MS):* Centrifuged thiochrome assay samples were further filtered through Costar® Spin-X® 0.22 µm filters via centrifugation at 14500 rpm, 1 min and 10 µL of filtrates were injected for analysis. The LC method used on Exion-LC series UHPLC (Shimadzu) was the same as mentioned in the previous section with 30 µL of injection volume. Following parameters were utilized in the MS method on SciEX-X500LR-QTOF system – positive ESI mode with + 3500 V ion spray voltage; MRM-HR method; 35 V collision energy; and 10 V de-clustering potential at 300°C. Masses (m/z) of HMP, THZ, and thiamin thiochrome parent ions used for detection were 140.0815, 144.0479, and 263.0888 respectively, and those for fragment ions were 122.0718, 113.0297, and 122.0718 respectively.

3.2.5 Analyzing the ratio of mutants in co-cultures: For calculating the ratio of two strains present in the co-culture over time, we correlated the OD_{600} and fluorescence values for the fluorescently tagged strains grown in the P2 passage without thiamin. To start with, we generated standard curves of fluorescence at $485_{ex}/535_{em}$ against OD_{600} for GFP-tagged strains. Cells were then grown in P2 passage either as monocultures or as co-cultures with all four possible pairwise combinations of the *ΔthiC::kan*^{R} strain or the *ΔthiC** strain with the *ΔthiE::kan*^{R} strain or the *ΔthiE** strain i.e. *ΔthiC*-thiE* (*C*E*), *ΔthiC-thiE** (*CE**), and controls *ΔthiC-thiE* (*CE*) and *ΔthiC*-thiE** (*C*E**). Values of the total fluorescence of *C*E* and *CE** co-cultures carrying a single fluorescent strain were then normalized using the *CE* co-culture a control, and the OD_{600} value of that fluorescent strain was calculated using the fluorescence v/s OD_{600} correlation of that strain. The OD_{600} of untagged strain was then obtained by subtracting the OD_{600} of the tagged strain from the total OD_{600} of the co-culture. From this exercise, we obtained ratios of strains in *ΔthiC-thiE* and *ΔthiC-ΔthiG* co-cultures over time.

Sr. No.	Primer name/ purpose	Primer Sequence (5' → 3')
1.	*aidB_GFPmut2*_for	GAATGATTTATTGCTGCGGGCGACGGGGGGAGTGTGTGT GTAAGCGTATACCAACTCAGCTTCCTTTCG
2.	*aidB_GFPmut2*_rev	CAATTTTCACATATTTCATTTAGTTAATCGAAACCAGCGT CGCATCAGTCGATGAGCTCGAGATATGAATATC

Table 3.1 Sequences of primers used in Chapter 3. Key: for = forward and rev = reverse primer.

3.3 Research design, Results, and Discussion

3.3.1 Analyzing the growth of pairwise co-cultures of thi⁻ mutants in M9 minimal medium without thiamin supplementation

After studying the physiology of *thi*⁻ mutants, we analyzed the growth of their pairwise co-cultures *ΔthiC-ΔthiE* (*CE*), *ΔthiC-ΔthiG* (*CG*), and *ΔthiE-ΔthiG* (*EG*) in thiamin-deficient M9 medium in the P2 passage. We hypothesized that if *thi*⁻ mutants are capable of sharing thiamin or its biosynthesis intermediates, the co-cultures will survive in thiamin-deficient P2 in comparison to their respective monocultures. Figure 3.1 depicts all possible scenarios in which these co-cultures can survive by exchanging thiamin biosynthesis intermediates. We started these co-cultures in three different ratios – 1:9, 1:1 and 9:1, and observed that the 1:9 ratio of the *CE* co-culture and the *CG* co-culture showed an increased survival in comparison to the respective monocultures after 24 h of growth (Figures 3.2A and 3.2B). On the other hand, the *EG* co-culture did not show any difference in growth over the monocultures (Figure 3.2C).

The *CE* co-culture showed increased survival as compared to the pure cultures grown in thiamin-deficient P2, which likely indicates that HMP and thiamin can be exchanged in the medium. This is because, this co-culture could have grown only if the *ΔthiE* strain supplemented the *ΔthiC* strain with HMP, and the *ΔthiC* strain in return, supplemented the *ΔthiE* strain with thiamin (Figures 3.1A and 3.2A). Similarly, the *EG* co-culture could have grown only if the *ΔthiE* strain supplemented the *ΔthiG* strain with THZ, and the *ΔthiG* strain in return, supplemented the *ΔthiE* strain with thiamin (Figures 3.1B and 3.2C). Our feeding studies (Chapter 2) show that *E.*

coli is able to salvage both THZ and thiamin, and the results from the *CE* co-culture show that thiamin can be exchanged among *E. coli* mutants. Thus, probably the only reason that the *EG* co-culture does not survive is because THZ is not being released by the *ΔthiE* strain in sufficient concentrations such that the *ΔthiG* strain can salvage it and grow. The *CG* co-culture also showed a marked increase in their growth over the monocultures (Figures 3.1C and 3.2B). This co-culture could have grown using three distinct possibilities as explained earlier (Figure 3.1C). As the data of the *EG* co-culture points to THZ not getting exchanged, only the third possibility holds true for the *CG* co-culture, which is that HMP and thiamin are exchanged among these two mutants.

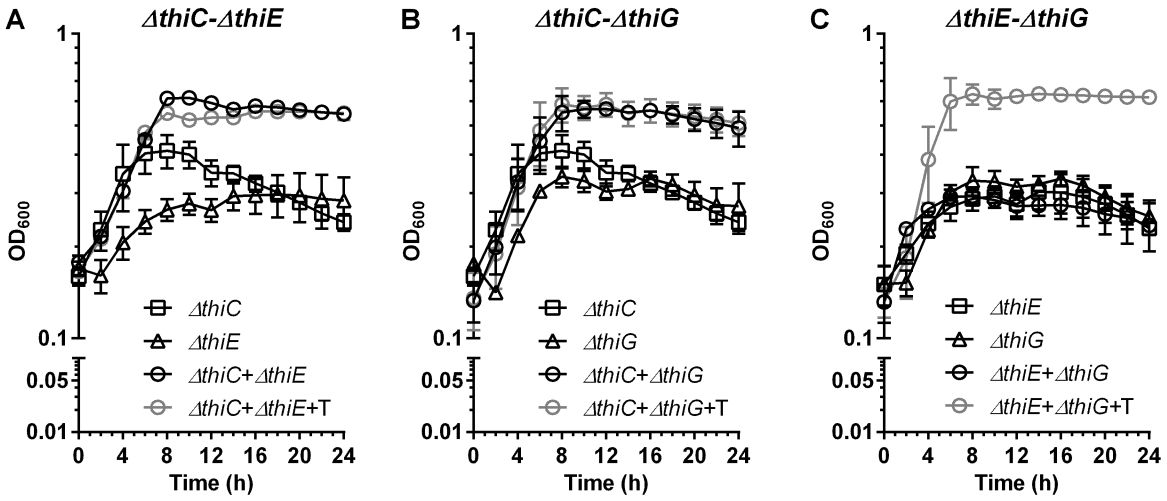

Figure 3.2 Co-cultures of thiamin mutants of *E. coli*. (A) *CE*, (B) *CG*, and (C) *EG* co-cultures in the P2 passage. + T = with thiamin. Means ± standard errors of means from three independent experiments are plotted. Sathe *et al*, J Bacteriol, 2022.

These results obtained are confirmed by several observations in literature which report prevalent exchange of HMP and thiamin analogues in comparison to the THZ moiety[9–14]. Presence of free HMP, HMP auxotrophs, and HMP-releasing microorganisms in natural ecosystems has also been widely reported[9,10,15,16]. In addition, various primary and secondary transporters have

been characterized for HMP, thiamin, and their analogues, but only putative transporters have been associated with the function of THZ transport[17–21].

3.3.2 Analyzing changes in co-culture dynamics with changing carbon sources

As thiamin is involved in the metabolism of pyruvate, but not in that of succinate, the intermediate in the Krebs cycle after α-ketoglutarate, we analyzed the role of these carbon sources (C-source) on the growth of *thi*$^-$ mutants and their co-cultures. In the medium containing pyruvate as the sole C-source instead of glucose (M9-pyruvate medium), thiamin requirement for *thi*$^-$ mutants would increase as decarboxylation of pyruvate would have to be the first step for its utilization. Thus, we hypothesized that *thi*$^-$ mutants and subsequently their co-cultures would have lower growth in M9-pyruvate medium. On the other hand, in the medium containing succinate as the sole C-source (M9-succinate medium), the requirement for thiamin would be lesser as the intermediate after thiamin-catalyzed steps of Krebs cycle is being provided. Thus, in this medium, we hypothesized that *thi*$^-$ mutants and their co-cultures would grow better in comparison to M9-glucose medium. Indeed, *CE* and *CG* co-cultures grew in M9-pyruvate and M9-succinate media without thiamin whereas the *EG* co-culture did not (Figure 3.3).

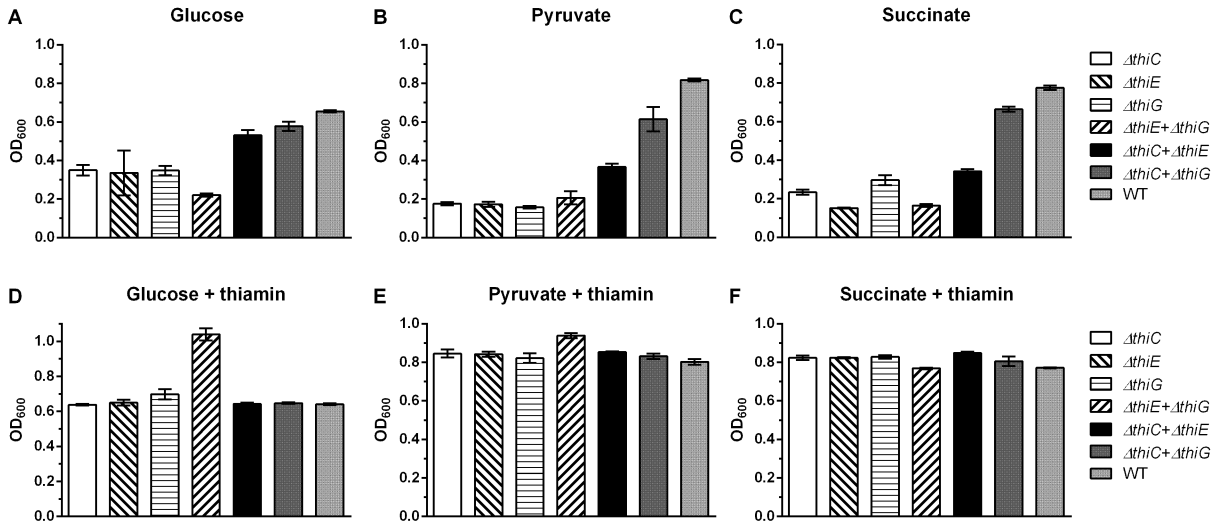

Figure 3.3 Growth phenotypes of cultures of thiamin biosynthesis mutants of *E. coli* with different carbon sources. Cultures with (A) glucose, (B) pyruvate, (C) succinate, (D) glucose and

thiamin, (E) pyruvate and thiamin, and (F) succinate and thiamin in the P2 passage. Means ± standard errors of the means from three independent experiments are plotted. Sathe *et al*, J Bacteriol, 2022.

We also observed that the *CG* co-culture showed better growth in all three media, but did not show significant differences in growth across these three media. As the *CE* co-culture did not survive well when pyruvate and succinate were used as C-sources, we used M9-glucose medium for all further experiments.

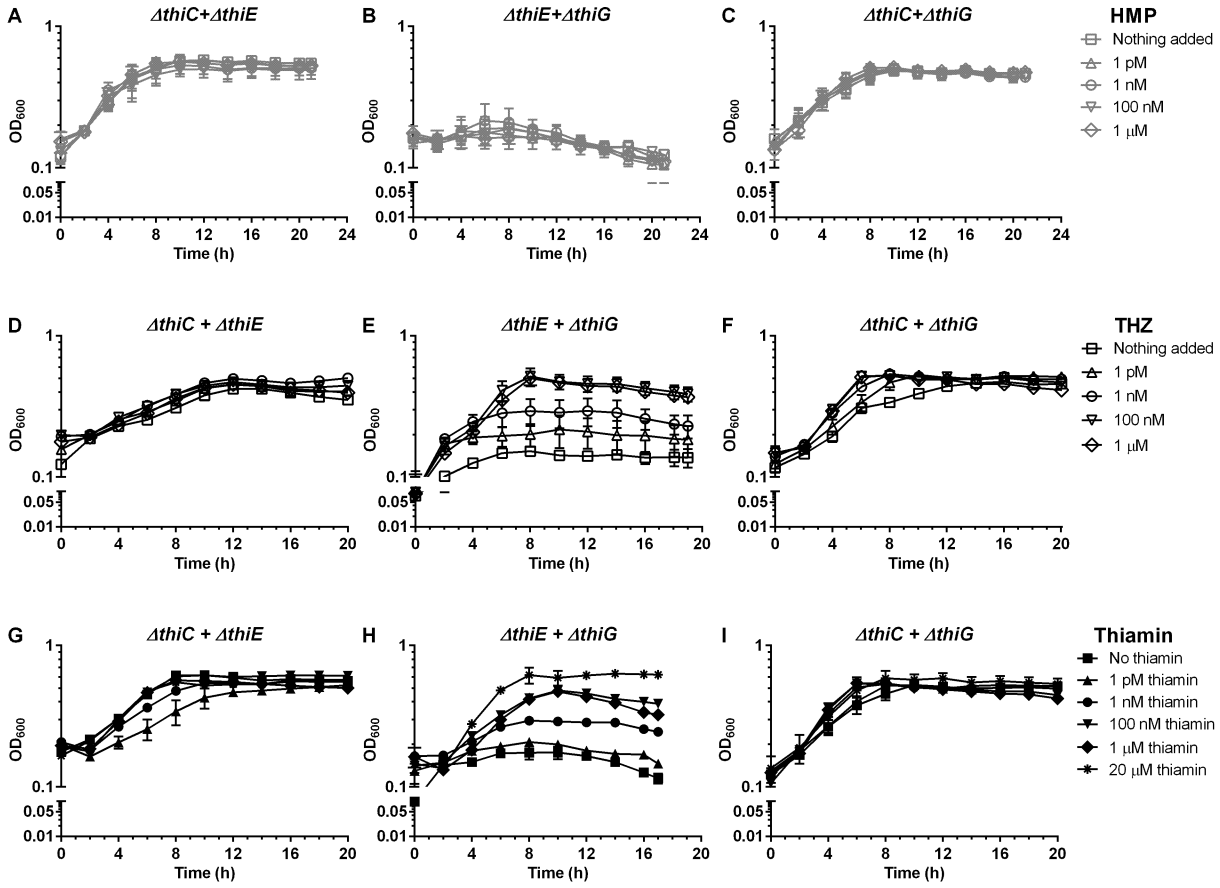

Figure 3.4 HMP, THZ, and thiamin supplementation to co-cultures of thiamin biosynthesis mutants of *E. coli*. Growth phenotypes of *CE*, *EG* and *CG* co-cultures on (A)-(C) HMP, (D)-(F)

THZ, and (G)-(I) thiamin supplementation. Empty grey, empty black and filled black symbols represent HMP, THZ, and thiamin, respectively. Symbols represent the following concentrations: □ = Nothing added, Δ = 1 pM, ○ = 1 nM, ∇ = 100 nM, ◊ = 1 μM, * = 20 μM. Means ± standard errors of means from three independent experiments are plotted. Sathe *et al*, J Bacteriol, 2022.

3.3.3 Analyzing the effect of HMP, THZ and thiamin supplementation on co-cultures

We next supplemented HMP, THZ, and thiamin to co-cultures of *thi*$^-$ mutants. Both *CE* and *CG* co-cultures show growth with HMP and THZ as expected from their ability to survive even in the absence of thiamin (Figures 3.4A, B, D, and E). The *EG* co-culture showed concentration-dependent growth in the presence of THZ, but not with HMP (Figures 3.4C and F). Also, only the *ΔthiG* strain from the *EG* co-culture supplemented with THZ grew as verified by using the GFP-tagged strain of *ΔthiG* (*ΔthiG**) for the *EG* co-culture, which shows increased fluorescence and OD_{600} upon supplementation (Figure 3.5). This result reiterates that *E. coli* has differential ability to pick up different thiamin biosynthesis pathway intermediates, which improve its growth in a concentration-dependent manner.

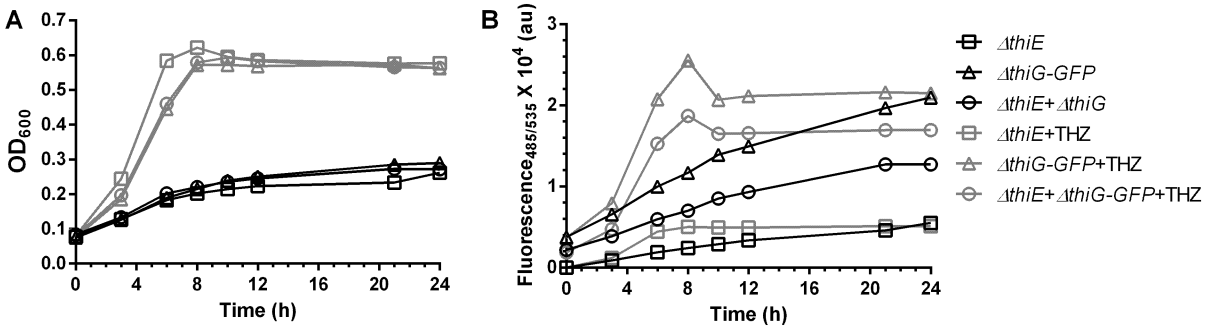

Figure 3.5 THZ supplementation to co-cultures and monocultures of *ΔthiE* and *ΔthiG-GFP* mutants of *E. coli*. (A) OD_{600} and (B) fluorescence at 535 nm for monocultures and co-cultures. Average values from two independent experiments are plotted.

In addition, *CE* and *CG* co-cultures started at a total starting OD_{600} of 0.01 do not survive in the absence of thiamin (Figure 3.6). This result further corroborates the importance of enough thiamin biosynthesis intermediates being released from donor cells at the beginning of the co-culture for its survival., and is also reminiscent of the quorum sensing behaviour of cells.

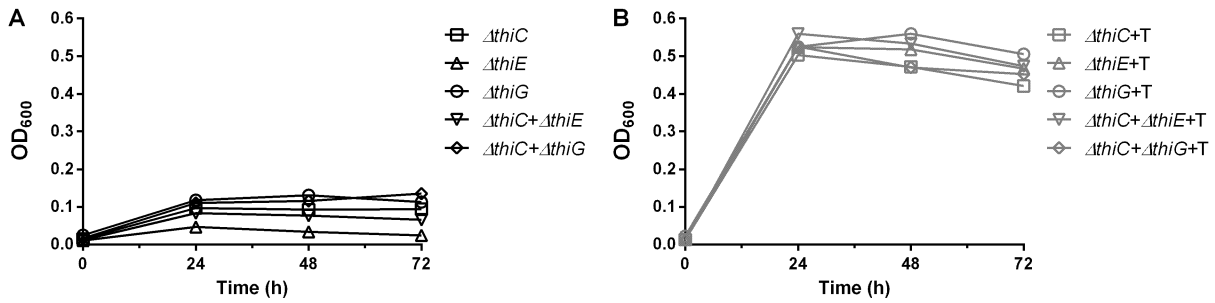

Figure 3.6 Co-cultures of thiamin mutants of *E. coli* started at 0.01 initial OD_{600}. Co-cultures of thiamin mutants in (A) the absence and (B) the presence of thiamin. Average values from two independent experiments are plotted.

3.3.4 Detecting thiamin, HMP, and THZ produced de novo by CE and CG co-cultures

We hypothesized that *CE* and *CG* co-cultures survive due to *de novo* production of thiamin. Thus, lysates and spent media from co-cultures and monocultures of *thi*$^-$ mutants were subjected to thiochrome assay which converts thiamin and its phosphorylated analogues thiamin monophosphate (ThMP) and thiamin diphosphate (ThDP) to their fluorescent derivatives, thiochrome and thiochrome phosphates[8]. We observed that the ThDP standard dephosphorylated due to harsh alkaline and acidic treatments used in the assay, leading to the detection of only its dephosphorylated analogues – thiochrome monophosphate and thiochrome (Figure 3.7A, 3rd trace from bottom). Further, data showed that the amount of thiochrome monophosphate present in lysates of *CE* and *CG* co-cultures was significantly higher than their respective monocultures when measured at 24 h, similar to the amount present in the WT cell lysate (Figure 3.7B).

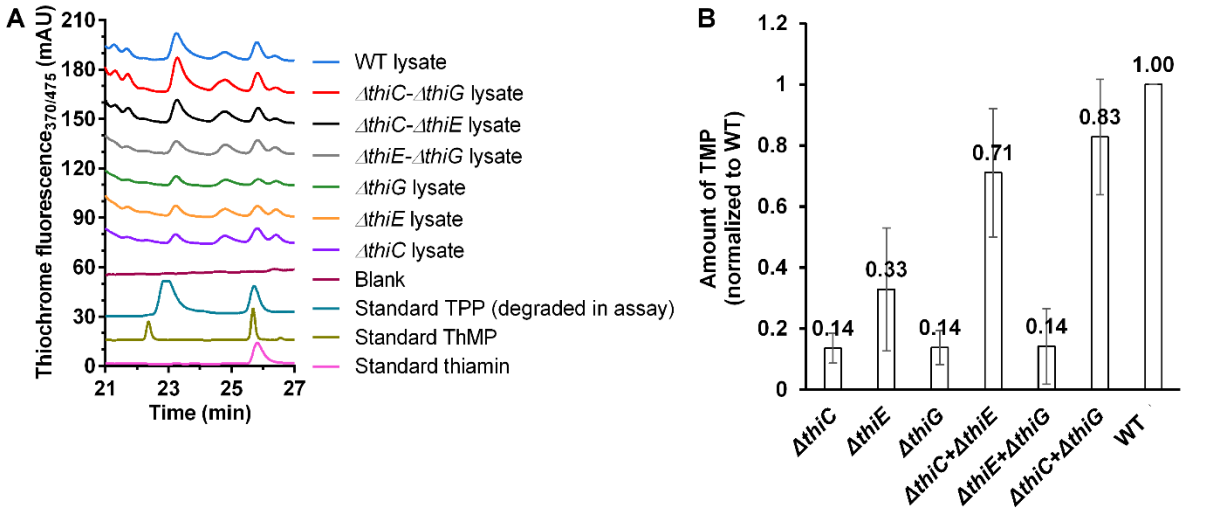

Figure 3.7 HPLC analysis of lysates of *E. coli* cultures treated with thiochrome assay. Lysates of thiochrome-treated P2 cultures as analyzed by (A) HPLC chromatogram and (B) quantitation of the amount of thiochrome monophosphate in lysates normalized to that in the WT strain. Means ± standard errors of the means from three independent experiments are plotted. Sathe *et al*, J Bacteriol, 2022.

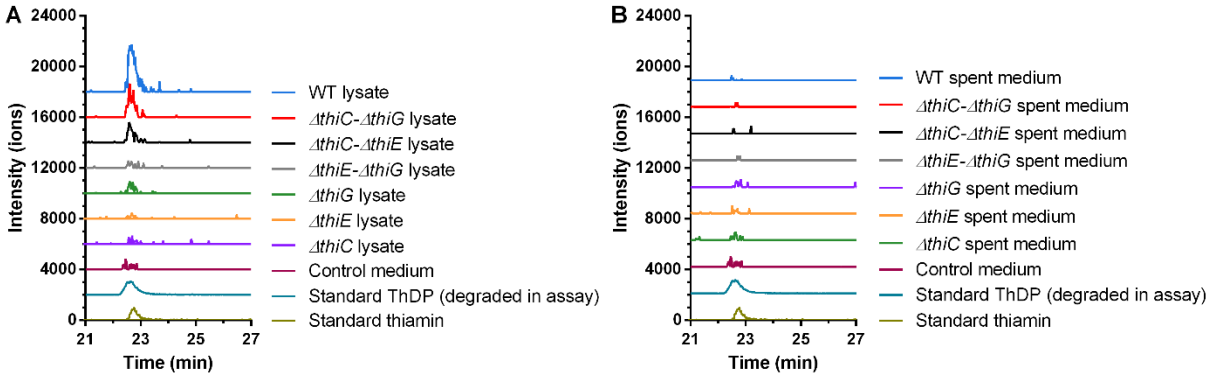

Figure 3.8 LC-MS/MS analysis of *E. coli* cultures treated with thiochrome assay. Analysis of (A) lysates and (B) spent media from cultures. The peak at 22.75 min was extracted using the m/z value of 263.0888 for thiamin thiochrome.

On the other hand, the amount detected from lysates of the *EG* co-culture and its respective monocultures was similar and lower than that present in the WT lysate. LC-MS/ MS analysis for detection of thiochrome monophosphate also showed its presence in *CE* and *CG* co-cultures (Figure 3.8A). These results showed that *de novo* biosynthesis of thiamin occurs in *CE* and *CG* co-cultures, allowing their survival, whereas the *EG* co-culture is unable to grow due to its lack.

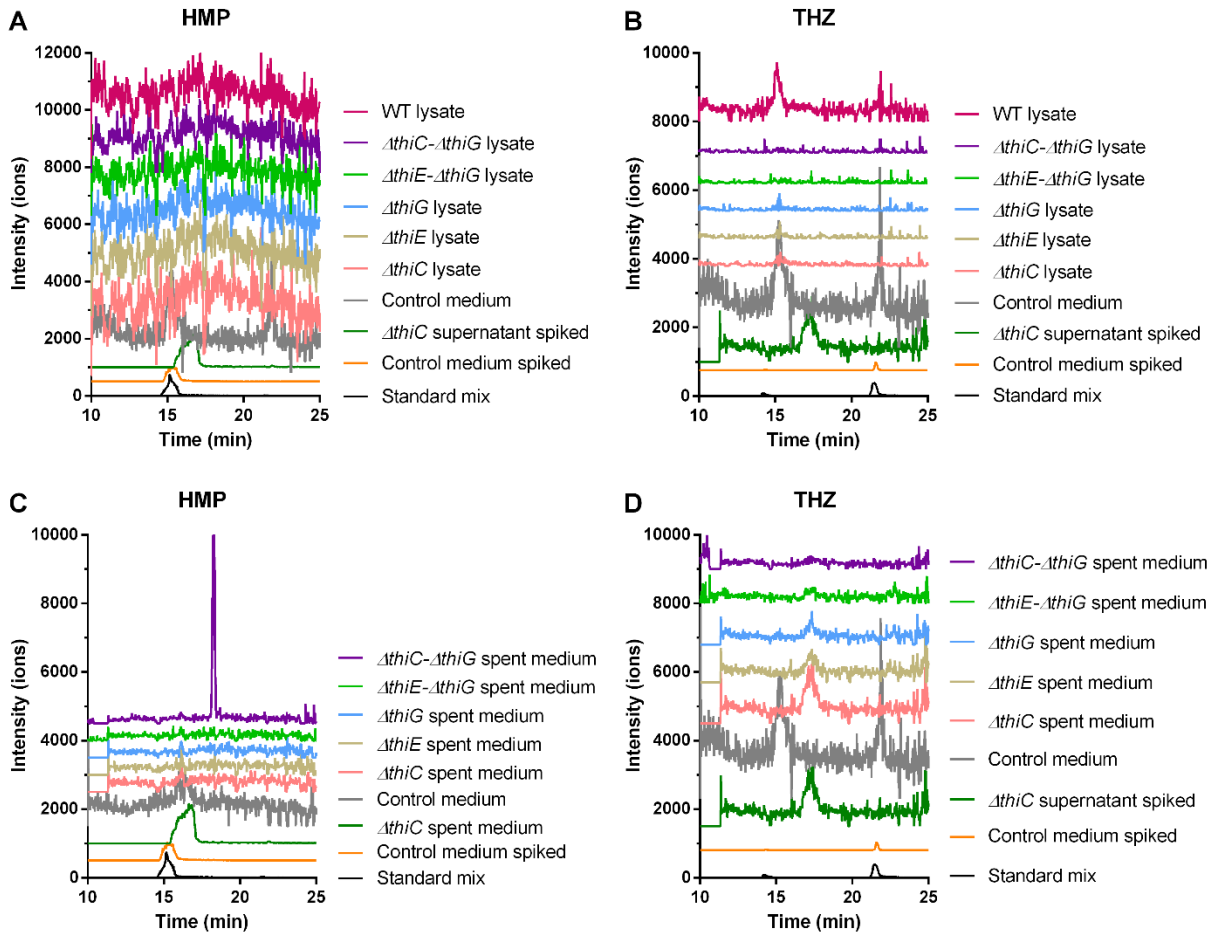

Figure 3.9 LC-MS/MS analysis of *E. coli* cultures for HMP and THZ detection. Analysis of (A) and (B) lysates and that of spent media (C) and (D) for *E. coli* cultures.

The analysis of spent media from monocultures and co-cultures did not show presence of thiochrome as compared to the control medium (Figure 3.8B). Also, neither HMP nor THZ could be detected from either lysates or spent media of monocultures or co-cultures (Figure 3.9). This might be due to: (i) very low levels of thiamin biosynthesis intermediates exchanged by strains in *CE* and *CG* co-cultures that survive, (ii) dilution of spent media as compared to cell lysates thus reducing its intermediate concentration, and (iii) both HMP and thiamin being picked up immediately by cells as they are growth-limiting nutrients for strains in *CE* and *CG* co-cultures, thus reducing their concentration in spent media beyond detection. As *E. coli* grows when fed with THZ, HMP, and thiamin in nanomolar concentration, all three possibilities might hold true.

3.3.5 Calculating ratios of strains in CE and CG co-cultures

We next calculated ratios of individual strains in *CE* and *CG* co-cultures in P2 passage in the presence and absence of thiamin. We observed that ratios of mutants in *CE* and *CG* co-cultures change over a period of 24 h without thiamin supplementation (Figures 3.10 and 3.11). In the *C*E* co-culture (* denotes GFP-tag), the OD_{600} of the co-culture increased along with its fluorescence, indicating that the percentage of the tagged *ΔthiC* strain increases in the *CE* co-culture (Figures 3.10A and 3.10B). This result was further supported by those from the *CE** co-culture, wherein the OD_{600} of the co-culture increased but the fluorescence did not, showing that the percentage of the untagged *ΔthiC* strain increases (Figures 3.10C and 3.10D). Together, these results showed that the percentage of the *ΔthiC* strain in the co-cultures increased over time, to attain a ratio of about 8:2 at 24 h, of the *ΔthiC* strain to the *ΔthiE* strain in the co-culture (Figure 3.10E).

However, in the presence of thiamin, the ratios of the two strains do not change (Figures 3.10 and 3.11). The OD_{600} of the co-culture increases for both *C*E* and *CE** co-cultures, but the fluorescence of only the *CE** co-culture increases whereas that of the C*E co-culture does not (Figure 3.10A-D). Upon quantitation, we observed that the ratio of the *ΔthiC* strain to the *ΔthiE* strain stays at around 1:9, similar to the initial inoculation ratio (Figure 3.10E). Similar results were obtained for *CG* co-cultures in the presence and absence of thiamin (Figure 3.11). Also, ratios of strains did not deviate upon re-inoculation of co-cultures in the P3 passage for further 24 h from the values at which they stabilized in the P2 passage (Figure 3.12).

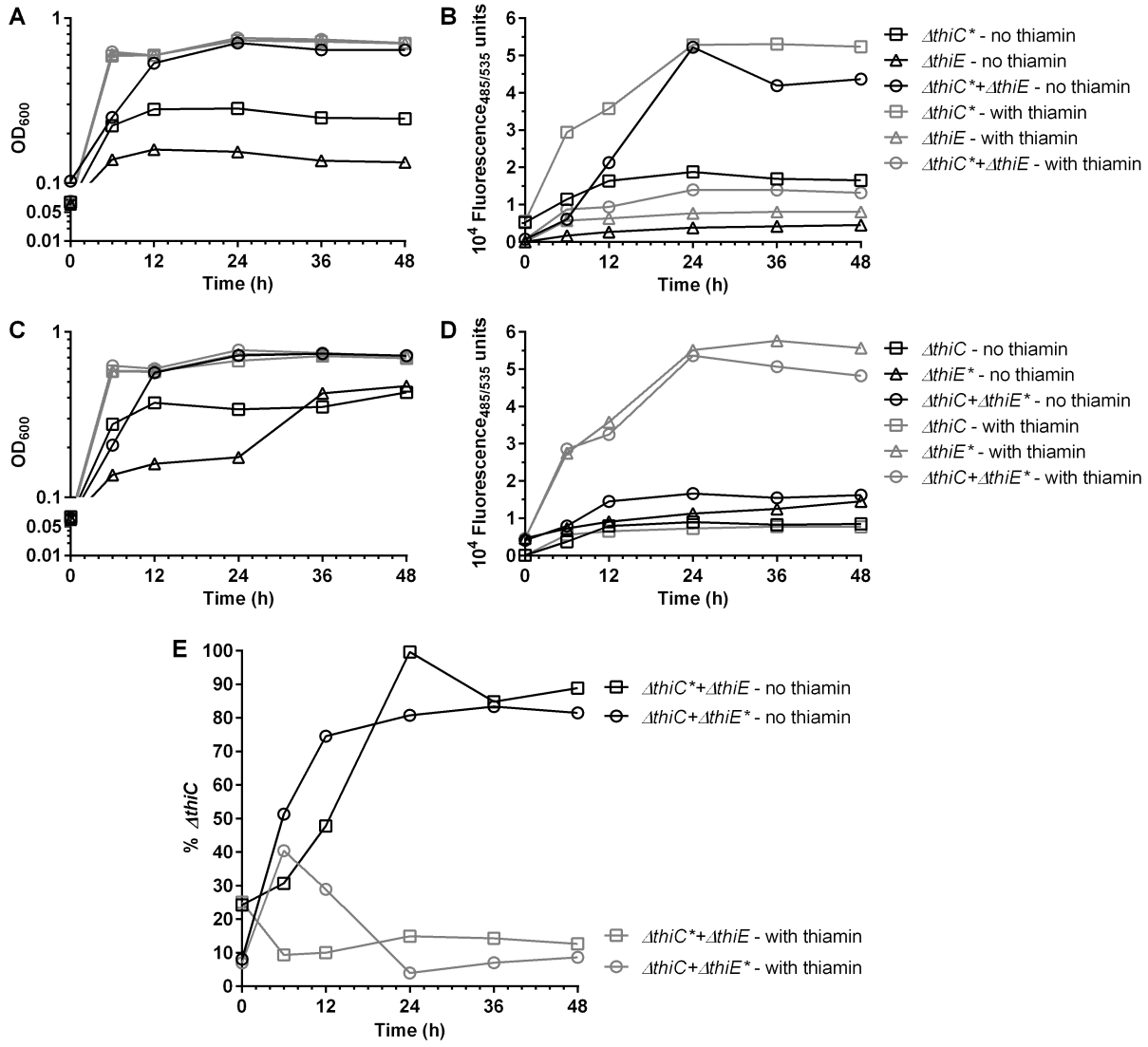

Figure 3.10 Quantitation of strains in the *CE* co-culture. Data for *C*E* (A) and (B), and *CE** (C) and (D) co-cultures. (A) and (C) OD_{600}, and (B) and (D) fluorescence at 535 nm. (E) *ΔthiC* strain percentage in co-cultures. Average values from two independent experiments are plotted. Sathe *et al*, J Bacteriol, 2022.

We hypothesize that the change in the ratio of strains in co-cultures in the absence of exogenously supplemented thiamin is due to dynamic changes over time in the release and uptake of HMP and thiamin. Additionally, we observed that the ratios do not alter in the P3 passage (third passage in M9) in either the presence or absence of thiamin, which might point to an equilibrium attained by the co-culture. This result also showed that the GFP label did not affect the final ratio of two strains in the co-culture. Taken together, these results suggest that when the nutrients are available in plenty in a community of auxotrophs, they might not interact with each other.

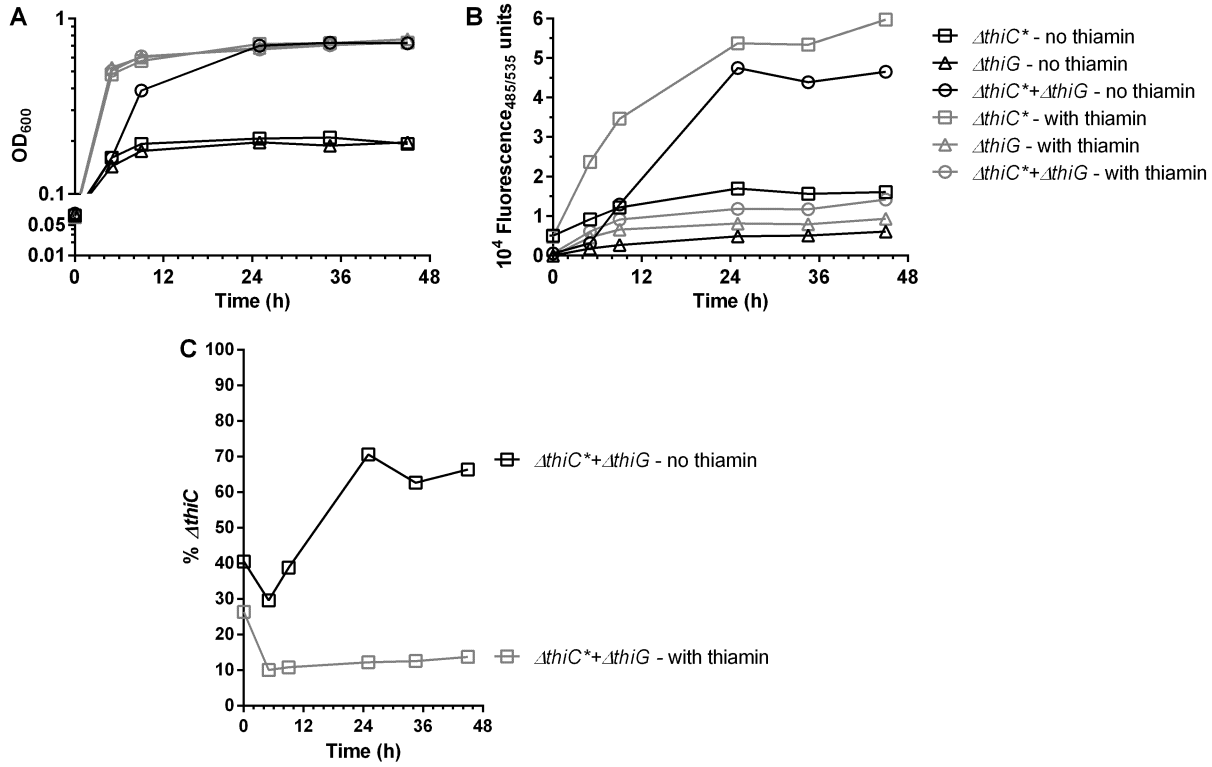

Figure 3.11 Quantitation of strains in the *C*G* co-culture. (A) OD_{600} and (B) fluorescence at 535 nm. (C) Percentage of the *ΔthiC* strain. Average values from two independent experiments are plotted. Sathe *et al*, J Bacteriol, 2022.

3.4 Summary

Data from various metagenomic, proteomic and metabolomics analyses has predicted the exchange of thiamin and its biosynthesis intermediates HMP, THZ, and their analogues in microbial communities[11,17,22–24]. Transporters for these metabolites have also been either predicted or characterized. To analyze the exchange of these intermediates in more detail, we co-cultured thiamin biosynthesis mutants of *E. coli*. Of the pairwise combinations, *CE* and *CG* co-cultures survive in the absence of thiamin in M9 minimal medium, whereas the *EG* co-culture does not. We also observed that thiamin monophosphate amounts in *CE* and *CG* co-cultures are similar to that of the WT strain, showing that they survive due to *de novo* synthesis of thiamin. This showed that HMP and thiamin can be exchanged in *E. coli*, whereas the exchange of THZ is restricted.

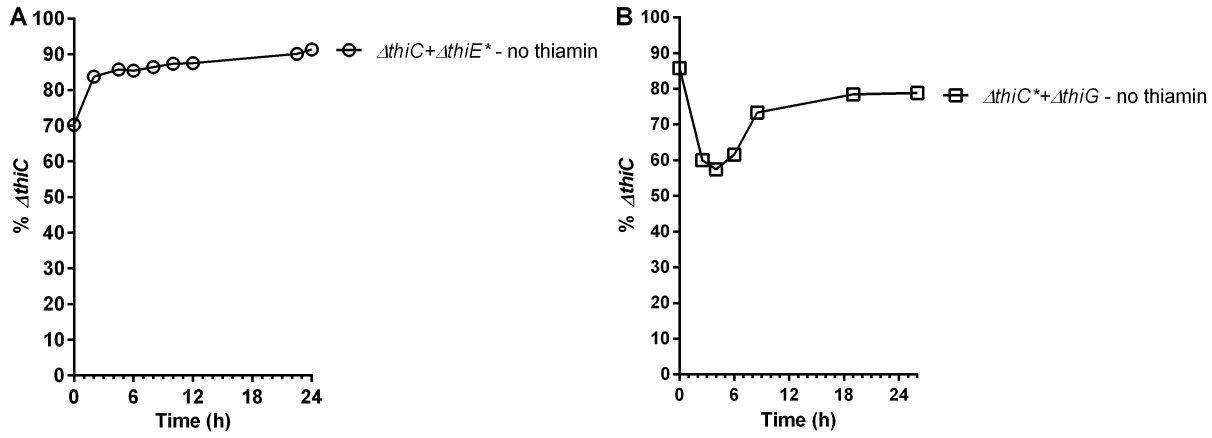

Figure 3.12 Quantitation of strains in co-cultures after re-inoculation in passage P3. Data for (A) *CE** and (B) *C*G* co-cultures. Average values from two independent experiments are plotted.

Data from metagenomic analyses also shows that the number of HMP auxotrophs i.e. *thiC* auxotrophs in marine and gut microbiota is higher than the number of THZ auxotrophs i.e. *thiG*/ *thi4* (eukaryotic homologue of the *thiG* gene) auxotrophs[11,15]. For example, in marine ecosystem,

it has been observed that the ratio of *thiC:thiG+thi4* genes is between 0.06-0.28, less than one[15]. Another study reports that the ratio of *thiC* genes to *thiG* genes is less than one at depths of 0-80 m[10]. Out of 2,228 genomes studied for the human gut microbiome (HGM) project, the number of HMP auxotrophs banked at 199, but only 114 THZ auxotrophs were reported[11]. Apart from this, more transporters have been characterized for thiamin and HMP analogues, whereas only a couple of THZ transporters have been predicted for THZ[18,19,24–26]. Thus, the data obtained from our experiments together with these reports points to HMP being the predominantly shared thiamin biosynthesis intermediate among thiamin auxotrophs (Figure 3.13A).

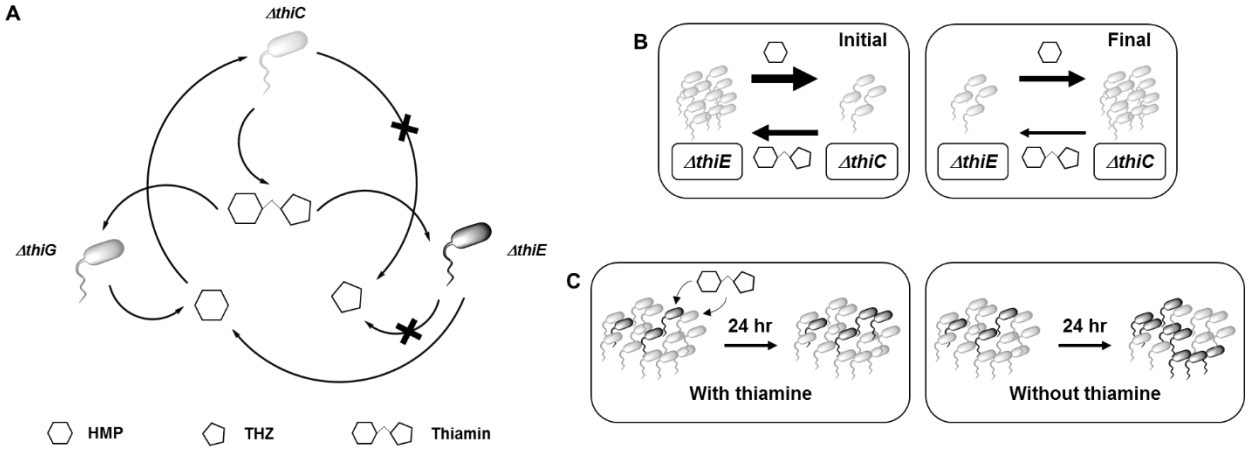

Figure 3.13 Models depicting possibilities of exchange and changes in ratios. (A) Exchange of intermediates among *thi*$^-$ mutants of *E. coli*. (B) Exchange of intermediates at initial and final stages of *CE* co-culture without thiamin supplementation. (C) Changes in ratios of mutants in the *CE* co-culture after 24 h of growth with or without thiamin supplementation.

We also observed that the ratio of thiamin mutants in co-cultures changes over time in the absence of thiamin, whereas it does not alter when thiamin is available in the medium. We attribute this to the dynamic exchange of HMP and thiamin by the two strains, wherein, the ratio stabilized at ~8:2 of the *ΔthiC* strain to the *ΔthiE* or the *ΔthiG* strain at the end of 24 h in the absence of

exogenous thiamin supply (Fig 3.13B). For example, at the beginning of the *CE* co-culture, the number of *ΔthiE* cells needs to be higher than that of the *ΔthiC* cells such that initial transfer of HMP happens to the *ΔthiC* cells, which can then make thiamin and supplement it back to the *ΔthiE* cells. As thiamin is required in catalytic amounts, *ΔthiC* cells can survive with the thiamin pool generated, and might only need an intermittent supply of HMP to regenerate that pool, thus supporting the growth of some *ΔthiE* cells. This almost reverses the ratio of these two strains in the co-culture, a phenomenon also observed for the *CG* co-culture.

Alternately, when thiamin is available in plenty, each individual strain in the co-culture can survive by salvaging it directly from the medium, and hence the ratio does not change much, owing to a lack of interaction (Fig 3.13C). Similar phenomenon has been observed before where seasonal blooms of auxotrophs in a marine environment are known to alter the concentration of thiamin biosynthesis intermediates, whereas with a lower microbial load, their concentration remained similar[16]. Also, a change in the ratio of auxotrophs in a co-culture involved in exchange of thiamin or other metabolites has been observed when grown under nutrient-deficient conditions[12,27,28].

The prevalent exchange of HMP and its analogues over that of THZ might be explained on the basis of THZ biosynthesis involving several steps, intermediates and enzymes, as opposed to HMP biosynthesis[1]. HMP is synthesized in a single step rearrangement reaction by the enzyme ThiC, whereas THZ is assembled by the enzyme ThiG using three intermediates – 1-deoxyxylulose-5-phosphate (DXP), ThiS-thiocarboxylate, and dehydroglycine. Each of these three intermediates have intriguing properties in terms of metabolism. The biosynthesis of DXP itself requires thiamin as a cofactor[29]. The sulfur atom involves a relay of sulfur-transfer proteins, which finally gets installed in the THZ ring, using an enzyme ThiS, whose sole function seems to be this S-transfer[30]. Dehydroglycine is an unstable intermediate that can be stabilized only in the enzyme active site[31]. Additionally, the eukaryotic homologue of ThiG, the Thi4 enzyme, is a suicide enzyme as the S-atom from its own active site cysteine is utilized for THZ biosynthesis[32]. On the contrary, HMP biosynthesis uses a single intermediate AIR which is a shared intermediate on vitamin B_1, purine, and vitamin B_{12} biosynthesis pathways[33–35]. Thus, a higher metabolic cost might be involved in the biosynthesis of THZ in comparison to that of HMP.

3.5 References

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Chapter 4: Analyzing the cross-talk between thiamin and purine biosynthesis mutants of *Escherichia coli* K-12 MG1655

4.1 Thiamin and purine biosynthesis pathways in *E. coli* share a common intermediate

The intermediate HMP in the thiamin biosynthesis pathway is synthesized by the enzyme ThiC from 5'-Phosphoribosyl-5-aminoimidazole (AIR), which is also an intermediate shared by the purine biosynthesis pathway in *E. coli* (Figure 4.1)[1,2]. As these two pathways are connected via a common intermediate, we hypothesized that the mutants of these two pathways might engage in a cross-talk that depends on exchange of specific metabolites. We also hypothesized that the flux through one of these pathways might affect the other pathway in these mutants, thus affecting the intermediates that are exchanged. Hence, we decided to analyze the cross-talk between purine and thiamin biosynthesis pathway mutants of *E. coli*.

AIR is synthesized by the enzyme PurM and is further converted to 5-carboxyamino-1-(5'-phosphoribosyl)imidazole (NCAIR) by PurK[3–5]. NCAIR is further used as a substrate to synthesize inosine and subsequently purines by other enzymes on the purine biosynthesis pathway[2,4]. Thus, the *ΔpurM* mutant of *E. coli* which is deficient in the biosynthesis of AIR is not able to produce both inosine and thiamin, and thus requires both these nutrients for its growth in a minimal medium. The *ΔpurK* mutant, on the other hand, can produce AIR, but cannot convert it to NCAIR, and thus is deficient in the biosynthesis of purines, but not that of thiamin.

We hypothesize that as the *ΔpurK* mutant produces AIR, but cannot convert it to NCAIR, it might accumulate AIR, which might result in subsequent accumulation of HMP in this strain. HMP accumulation might lead to the increased biosynthesis of thiamin in this mutant if the THZ biosynthesis in it is also upregulated to a similar extent. Thus, the *ΔpurK* mutant might be able to supplement the growth of the thiamin mutants in return for a purine in a minimal medium as depicted in Figure 4.2, hypotheses (i), (ii) and (iii). The *ΔpurK* mutant might be able to supplement either HMP or thiamin or both to the *ΔthiC* mutant in a co-culture (termed as the *CK* co-culture) in a minimal medium as shown in Figure 4.2, hypothesis (i). The co-culture of the *ΔpurK* mutant and the *ΔthiE* mutant (termed as the *EK* co-culture) might survive in a minimal medium only if

thiamin is provided by the *ΔpurK* mutant to the *ΔthiE* mutant in exchange for a purine Figure 4.2, hypothesis (ii). THZ transfer is not considered in the hypothesis (iii) in Figure 4.2 among the *ΔpurK* mutant and the *ΔthiG* mutant in a co-culture (termed as the *GK* co-culture) as it was shown in Chapter 3 that the exchange of THZ in a minimal medium is not feasible among *E. coli* mutants.

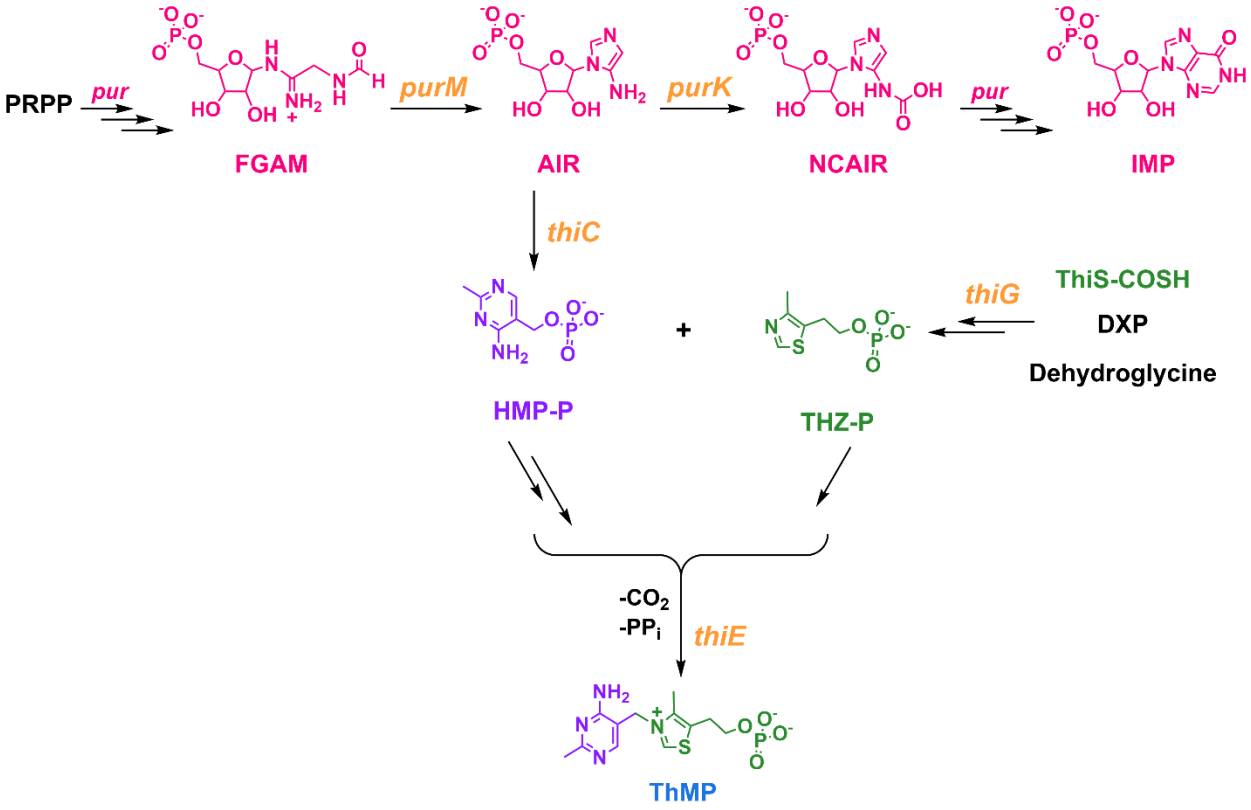

Figure 4.1 Purine and thiamin biosynthesis pathways in *E. coli*. Purine and thiamin biosynthesis pathways in *E. coli* diverge from their common intermediate 5'-Phosphoribosyl-5-aminoimidazole (AIR). Abbreviations: PRPP, Phosphoribosyl pyrophosphate; NCAIR, 5-carboxyamino-1-(5'-phosphoribosyl)imidazole; IMP, Inosine monophosphate; HMP-P, 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate; THZ-P, 4-methyl-5-(2-hydroxyethyl)thiazole phosphste; DXP - 1-deoxyxylulose-5-phosphate.

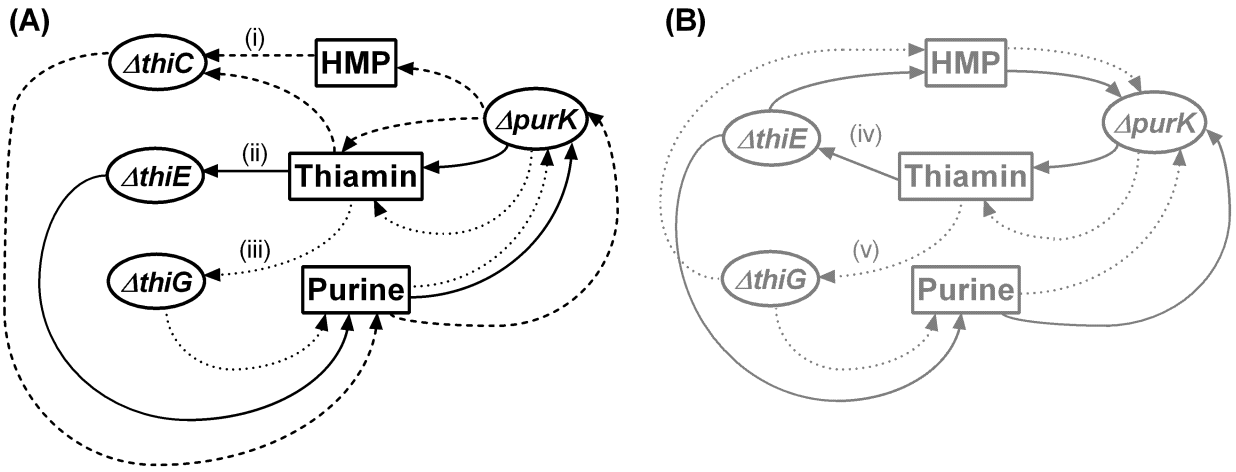

Figure 4.2 Models for exchange of intermediates among purine and thiamin biosynthesis mutants of *E. coli*. Models showing nutrients exchanged among thiamin biosynthesis mutants and (A) the *ΔpurK* mutant in black and (B) the *ΔpurM* mutant in gray colour. Exchange with (i) the *ΔthiC* mutant denoted by dashed arrows, (ii) and (iv) the *ΔthiE* mutant denoted by solid arrows, and (iii) and (v) the *ΔthiG* mutant denoted by dotted arrows.

The *ΔpurM* mutant, on the other hand, requires both a purine and either thiamin or HMP for its survival. Thus, a co-culture of the *ΔpurM* mutant and the *ΔthiC* mutant (termed as the *CM* co-culture) will not be able to survive in a minimal medium as they do not have complementary growth requirements. A co-culture of the *ΔpurM* mutant with the *ΔthiE* mutant (termed as the *EM* co-culture) might survive only if the *ΔthiE* mutant supplements the *ΔpurM* mutant with a purine and HMP, and the *ΔpurM* mutant synthesizes thiamin using this HMP and supplements it back to the *ΔthiE* mutant, as depicted in Figure 4.2, hypothesis (iv). Using similar exchange strategies, a co-culture of the *ΔpurM* mutant with the *ΔthiG* mutant (termed as the *GM* co-culture) might survive in a minimal medium, as depicted in Figure 4.2, hypothesis (v).

In order to validate the growth phenotypes of purine and thiamin biosynthesis mutants and these hypotheses for exchanges of intermediates, we analyzed the growth of monocultures and co-cultures of purine and thiamin biosynthesis mutants in the presence and absence of either inosine

or thiamin or both in the M9-glucose liquid minimal medium. We also analyzed the effect of growing purine and thiamin biosynthesis mutants next to each other on solid M9-glucose minimal medium. In addition, we analyzed ratios of mutants in co-cultures in M9-glucose media.

We observed that out of the 6 pairs of co-cultures mentioned between purine and thiamin mutants, only the *CK* co-culture survived in the absence of externally supplied purine and thiamin in M9-glucose minimal medium. We also observed that in the presence of inosine, *EM* and *GM* co-cultures could survive in M9-glucose minimal medium. Thus, these results further corroborated the exchange of HMP and inosine or a purine intermediate in co-cultures of *E. coli* mutants. We also calculated the ratio of mutants present in the *CK* co-culture, and observed that it oscillates, indicating that the two mutants might compete for nutrients.

4.2 Materials and methods

4.2.1 Chemicals and reagents: Inosine was obtained from TCI chemicals and thiamin was obtained from Sigma-Aldrich. All other chemicals, reagents and kits used were obtained either from TCI chemicals, HiMedia or Sigma unless otherwise specified. The enzymes used for generating knockouts, diagnostic PCRs, and cloning were obtained from TaKaRa.

4.2.2 Strains and plasmids: *E. coli* K-12 MG1655 strains *ΔpurK::kan*^{R} and *ΔpurM::kan*^{R} (termed as *ΔpurK* and *ΔpurM*) were generated by Dr. Amrita Hazra in Dr. Michiko Taga's lab at the University of California, Berkeley. *E. coli* K-12 MG1655 strains *ΔthiC*, *ΔthiE*, *ΔthiG*, *ΔthiC**, *ΔthiE**, and *ΔthiG** (* denotes the presence of the *GFPmut2-kan*^{R} cassette) were constructed in this study (Chapters 2 and 3). The *E. coli* K-12 BW25113 strain containing the plasmid pKD46 and the *E. coli* K-12 BW25141 strain containing the plasmid pKD3 were a gift from Dr. Nishad Matange at IISER Pune. The *ΔpurK::kan*^{R} *ΔthiC::cam*^{R} and *ΔpurK::kan*^{R} *ΔthiG::cam*^{R} double mutants (termed as *ΔpurK ΔthiC* and *ΔpurK ΔthiG*, respectively), were generated by recombination using the λ Red Recombineering system, with steps for primer design, mutant generation, and verification carried out as previously described[6,7]. Detailed steps for primer design, generating *ΔthiC::cam*^{R} and *ΔthiG::cam*^{R} fragments using the pKD3 plasmid, recombination, mutant selection, and diagnostic PCRs were described in Chapter 2, Materials and Methods, Section 2.2.3, and primers used are listed in Table 4.1 in this chapter. The *ΔpurK* strain was made chemically competent and transformed with pMRE145 plasmid

from Addgene containing the mScarlet fluorescent gene (termed as *ΔpurK*$^{\#}$) by Vibhor Gaikwad (MS Thesis – Vibhor Gaikwad).

4.2.3 Growth conditions and media: *E. coli* K-12 MG1655 cells were grown either in LB or in M9-Glucose minimal medium at 37°C, 180 rpm[8]. Whenever necessary, the medium was supplemented with various components in small defined amounts as mentioned.

4.2.3.1 *Primary culture set-up (LB and P1 cultures):* LB and P1 cultures of all strains were set up in the same manner as previously described (Chapter 2, Materials and Methods, Section 2.2.4.1).

4.2.3.2 *Secondary culture set-up (P2 cultures):* P2 cultures and co-cultures were set up following the same washing protocol as previously mentioned (Chapter 2, Materials and Methods, Section 2.2.4.2). Following this, co-cultures of mutants in liquid M9-Glucose medium were set up using the same protocol as previously described (Chapter 3, Materials and Methods, Section 3.2.3.2). The medium was supplemented with or without either inosine (50 μM) or thiamin (20 μM) or both (total 4 conditions) unless mentioned otherwise.

4.2.3.3 *Spot assays (in P2):* Cells washed after the P1 passage were used for spot assays as P2 cultures on M9-Glucose agar plates, supplemented with or without either inosine (50 μM) or thiamine (20 μM) or both, with 1.5% w/v agar. All different strains were normalized to an OD_{600} of 1.0, which is considered to be the culture density of ~10^8 cells per mL for *E. coli* K-12 MG1655. These normalized cultures were then serially diluted to different culture densities from 10^7 – 10^2 cells per mL of the culture, and 10 μL of each of these serially diluted cultures were spotted on M9-Glucose agar plates on a single line at an interval of 1.5 cm, with two different strains spotted on different parallel lines running at a distance of either 1.6 cm or 1.2 cm from each other. This was to maintain a distance of either 0.8 cm or 0.4 cm (half of the former) between the two strains used in the assay, as the spot diameter is ~0.8 cm, and thus the spot radius being 0.4 cm for each spot. The plates were then incubated at 37°C, for 96 h.

4.2.4 Analyzing the ratio of mutants in co-cultures: OD_{600} and fluorescence correlation method as described in Chapter 3, Materials and Methods, Section 3.2.5 was used for calculating the ratio of two strains in the co-culture in the P2 passage over time. Fluorescence at $485_{ex}/535_{em}$ was used for quantitating the GFP-tagged strain.

Sr. No.	Primer name/ purpose	Primer Sequence (5' → 3')
1.	*thiC::cam*^{R}_for	TCATCCGTCGTCTGACAAGCCACGTCCTTAACTTTTTGGA ATGAGCTATGCATATGAATATCCTCCTTAG
2.	*thiC::cam*^{R}_rev	AGGTACAGGAGGAAAATCAGGCTGATACATCACGCTTCC TCCTTACGCAGTGTGTAGGCTGGAGCTGCTT
3.	*thiG::cam*^{R}_for	GGATGGCGACCAGATCCTGCTTTTTCAGGTTATTGCAGG GGGTTGAAATGCATATGAATATCCTCCTTAG
4.	*thiG::cam*^{R}_rev	CAGTTGTCGCCAGCGATCGCTGAAGGTTTTCATGCCGAT GCCTCCAGAAATGTGTAGGCTGGAGCTGCTTC
5.	*purK::kan*^{R}_conf_for	GTCTGAATGACTGGCGCAAA
6.	*purM::kan*^{R}_conf_for	GCTTTCCCTGTTAGAATTGC
7.	*thiC::cam*^{R}_conf_for	CGAAGGGAACAAGAGTTAATCTGC
8.	*thiG::cam*^{R}_conf_for	CAAACTGTTCACGAACTACTGGAG
9.	*kan*^{R}_conf_rev	TAATCAGCACCTGGCTGTCG
10.	*cam*^{R}_conf_rev	AGTTGTCCATATTGGCCACG

Table 4.1 Sequences of primers used in Chapter 4. Key: for = forward primer, rev = reverse primer, conf = diagnostic PCR (confirmatory) primer, kan = kanamycin, cam = chloramphenicol.

4.3 Research design, Results, and Discussion

4.3.1 Verifying knockout mutants of purine biosynthesis pathway of E. coli K-12 MG1655 and generating double mutants of purine and thiamin biosynthesis pathways

E. coli K-12 MG1655 *ΔpurK::kan*^{R} *ΔthiC::cam*^{R} and *ΔpurK::kan*^{R} *ΔthiG::cam*^{R} mutants were created using the protocol mentioned earlier. Diagnostic PCRs were carried out to verify these three double mutants, along with the *E. coli* K-12 MG1655 *ΔpurK::kan*^{R} and *ΔpurM::kan*^{R} single mutants using specific primers for each mutant. The WT strain used as a negative control for the colony PCR showed no product as it lacks the kanamycin cassette for the reverse primer to

bind, but deletion mutants *ΔpurK::kan*^{R} and *ΔpurM::kan*^{R} showed products of expected lengths at around 1200 bp for the kanamycin cassette (Figure 4.3A, Table 4.1 – primers 5, 6, and 9). The control reactions using either the *thiC* gene forward confirmation primer or the *thiG* gene forward confirmation primer with the chloramphenicol cassette reverse primer showed no products, whereas *ΔpurK::kan*^{R} *ΔthiC::cam*^{R} and *ΔpurK::kan*^{R} *ΔthiG::cam*^{R} mutants showed products of expected lengths at around 1200 bp for the chloramphenicol cassette (Figure 4.3B, Table 4.1 – primers 7, 8, and 10).

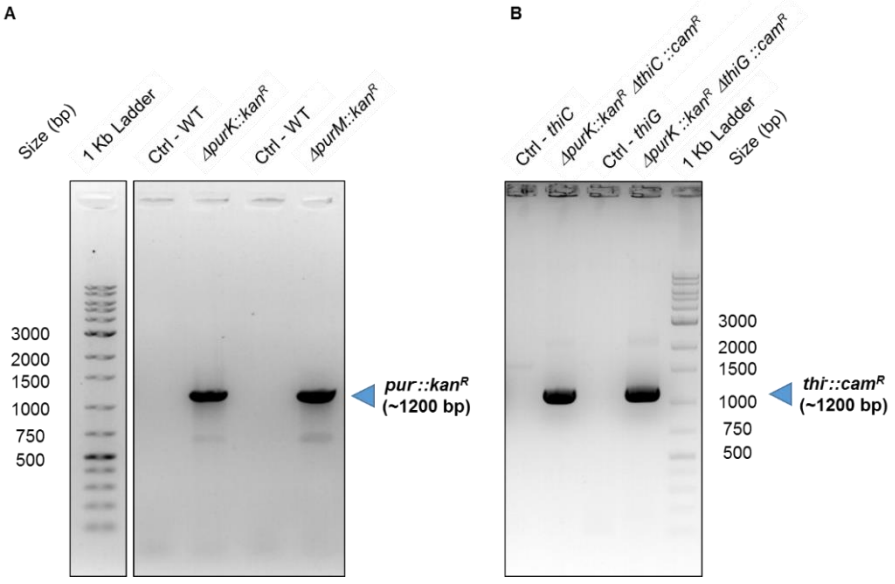

Figure 4.3 Diagnostic PCRs for verifying *E. coli* *ΔpurK::kan*^{R} *ΔthiC::cam*^{R} and *ΔpurK::kan*^{R} *ΔthiG::cam*^{R} double mutants and purine single mutants. PCR gels showing verification of (A) *pur*$^-$ gene deletions and (B) *thi*$^-$ gene deletions.

4.3.2 Growth phenotypes of purine biosynthesis mutants in LB

LB Miller broth was used to analyze growth phenotypes of *E. coli* K-12 MG1655 WT and *pur*$^-$ mutants. Growth rates of WT, *ΔpurK*, and *ΔpurM* strains were 1.653 ± 0.095, 1.508 ± 0.021, and 1.744 ± 0.143 h^{-1}, respectively in LB, which were comparable. This showed that these *pur*$^-$

mutants grew to the same extent as that of the WT strain in a rich medium, proving that they do not have any growth disadvantage as compared to the WT strain (Figure 4.4A and 4.4D).

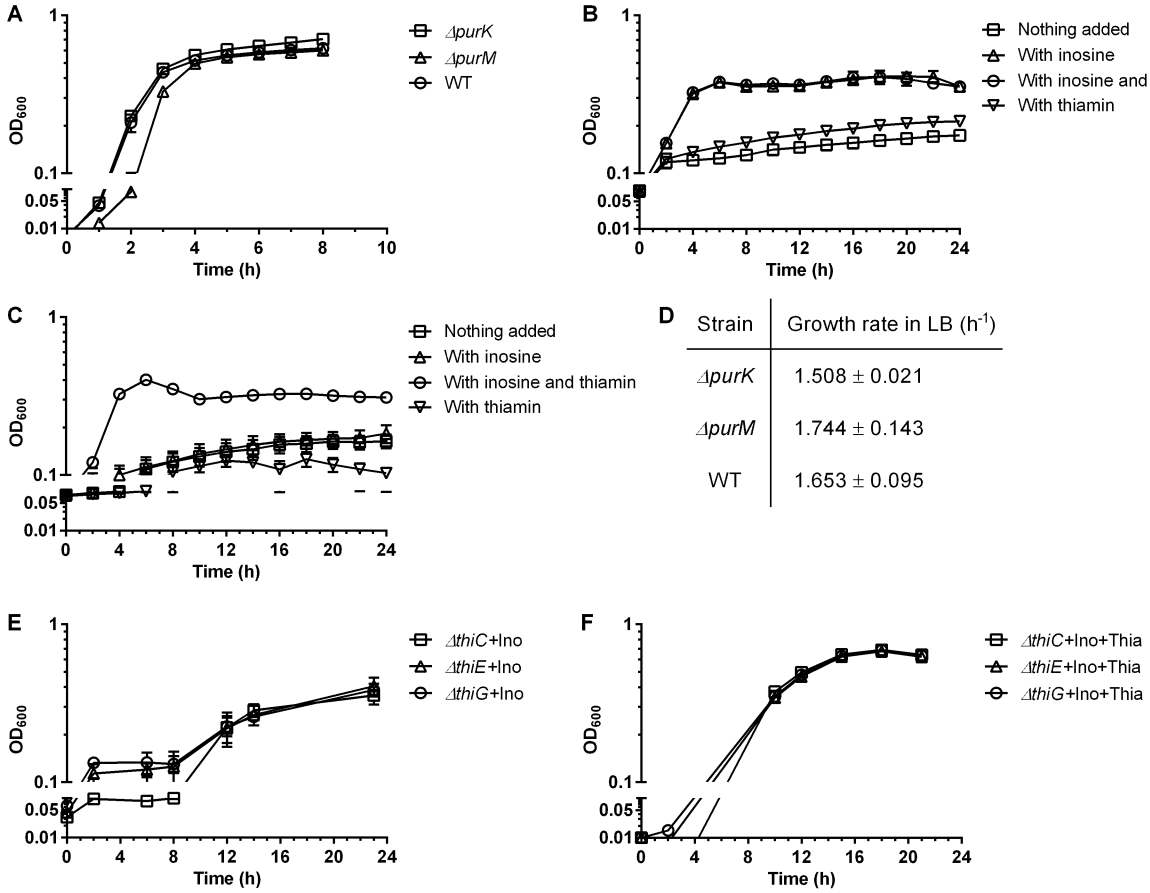

Figure 4.4 Growth phenotypes of *E. coli* purine biosynthesis mutants in LB and M9 medium. Means ± standard errors of the means from three independent experiments are plotted. (A) Growth phenotypes of *pur*⁻ mutants in LB. Growth phenotypes of (B) the *ΔpurK* mutant and (C) the *ΔpurM* mutant in P2 with and without inosine (50 µM) or thiamin (20 µM) or both. (D) Growth rates of *pur*⁻ mutants in LB. Growth phenotypes of *thi*⁻ mutants in P2 with (E) inosine (50 µM) and (F) both inosine (50 µM) and thiamin (20 µM).

4.3.3 Growth phenotypes of purine and thiamin biosynthesis mutants in M9-glucose minimal medium in the P2 passage

We next analyzed growth phenotypes of *thi*$^-$ mutants in M9-glucose minimal medium in the second passage P2. We observed that the *ΔpurK* mutant requires only inosine, whereas the *ΔpurM* mutant requires both inosine and thiamin for its survival (Figure 4.4B and 4.4C). We also supplemented thiamin mutants *ΔthiC*, *ΔthiE*, and *ΔthiG* with inosine or thiamin, and observed that they only require thiamin for their survival, but not inosine (Figures 4.4E, 4.4F, and Figure 2.3 – from Chapter 2). Taken together, these results show that purine and thiamin biosynthesis mutants of *E. coli* have distinct but complementary nutrient requirements.

4.3.4 The ΔthiC mutant grows next to the ΔpurK mutant on M9-glucose minimal agar supplemented with inosine

We next co-cultured the *ΔpurK* mutant of *E. coli* with the three thiamin biosynthesis mutants in a pairwise manner in the P2 passage, imaging every 24 h. When the *ΔthiC* mutant was grown on M9-glucose agar next to the *ΔpurK* mutant in the absence of inosine or thiamin, none of the mutants could supplement each other's growth, whereas, both the strains grew well upon supplementation of inosine and thiamin within 48 h (Figure 4.5A). In the presence of thiamin, only thiamin mutants survived on the solid medium, showing growth within 48 h (Figure 4.5A). But surprisingly, in the presence of inosine, the *ΔthiC* mutant started growing next to the *ΔpurK* mutant after 72 h (Figures 4.5A and 4.6A). The growth of the *ΔthiC* mutant in this case occurred 24 h after the growth of the *ΔpurK* mutant, and it grows towards the *ΔpurK* mutant as evident from the semicircular growth of the spot by the end of 96 h (Figure 4.6A). This shows that the *ΔpurK* mutant probably supplements either HMP or thiamin to the *ΔthiC* mutant, owing to which, it can survive next to the *ΔpurK* mutant. But *ΔthiE* or *ΔthiG* mutants spotted next to the *ΔpurK* mutant did not show any growth even after 96 h (Figures 4.5B-C and 4.6B-C). From our previous study in Chapter 3, we know that THZ is not exchanged in co-cultures of *E. coli* mutants, which leaves the possibility of only HMP or thiamin being exchanged[9]. Thus, the failure of *ΔthiE* or *ΔthiG* mutants to grow next to the *ΔpurK* mutant reduces the likelihood of thiamin being supplemented by the *ΔpurK* mutant, and rather points to HMP as the predominantly supplied nutrient.

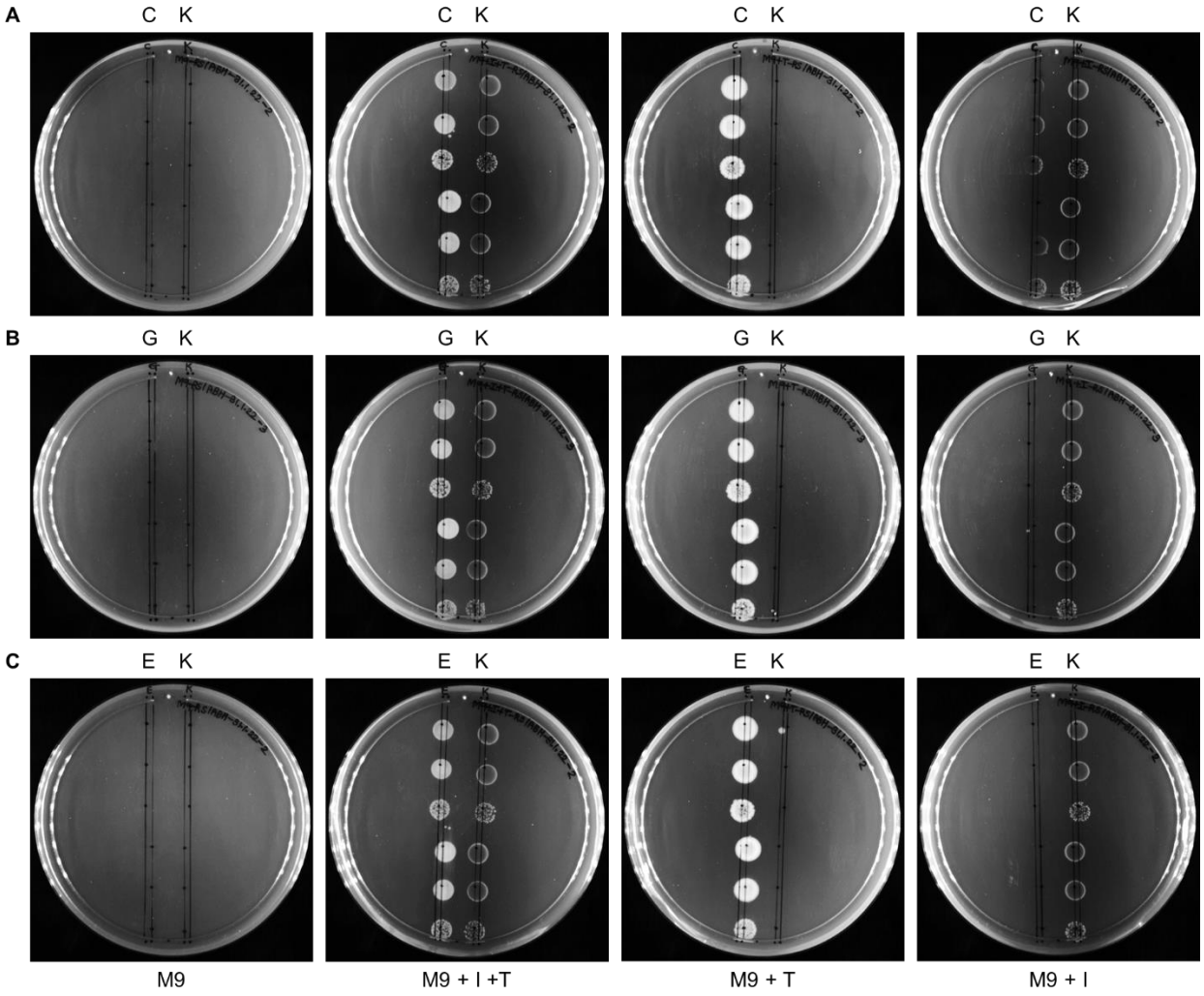

Figure 4.5 Spot assays at 72 h for purine and thiamin biosynthesis mutants of *E. coli*. Spot assays for *E. coli* purine and thiamin biosynthesis mutants grown next to each other on M9 minimal medium without or with supplementation of either inosine (50 µM) or thiamin (20 µM) or both, showing results at the end of 72 h in the P2 passage. Abbreviations: C - *ΔthiC*; K - *ΔpurK*; G - *ΔthiG*; E - *ΔthiE*. Growth of the *ΔpurK* mutant with (A) *ΔthiC*, (B) *ΔthiG*, and (C) *ΔthiE*.

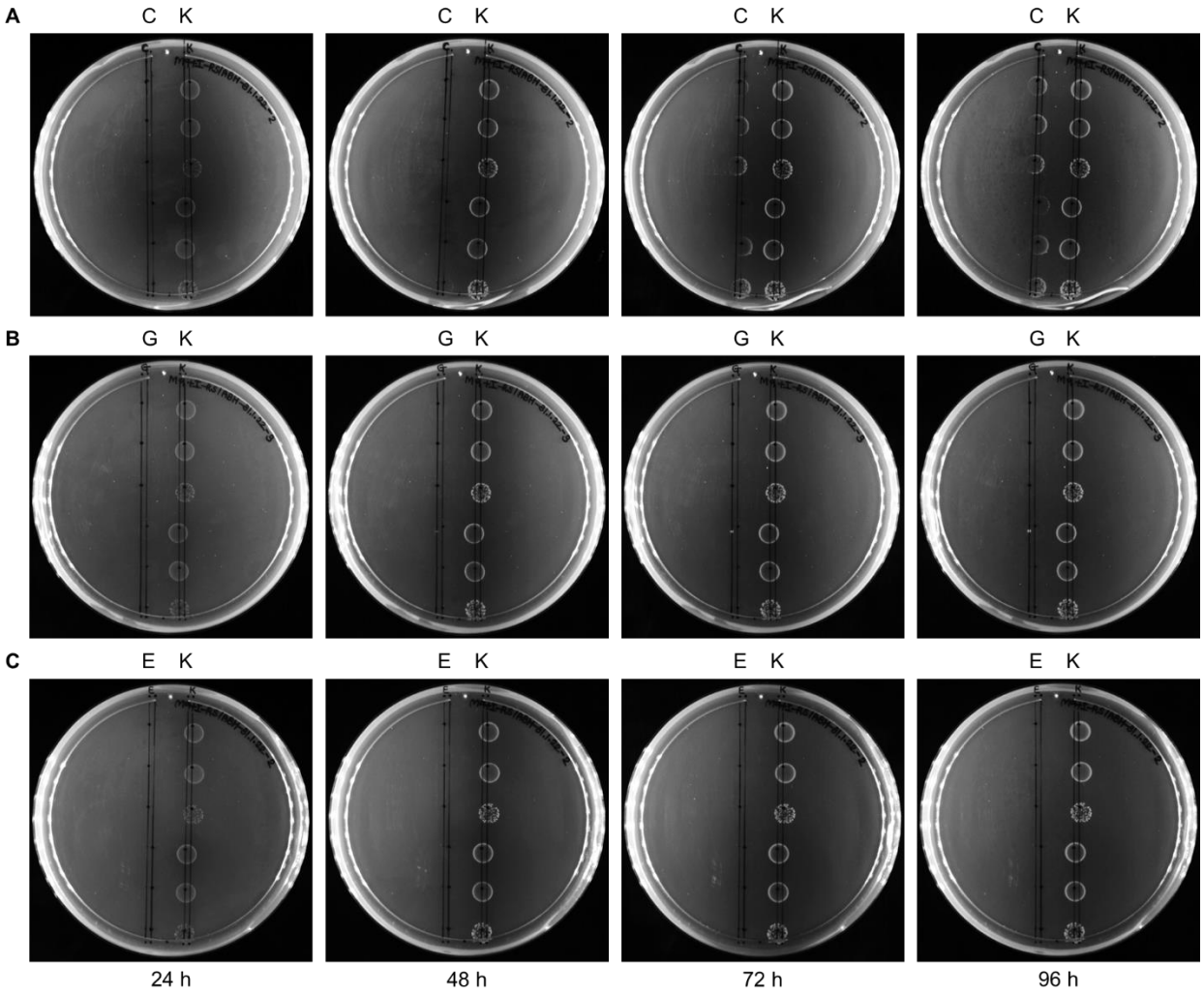

Figure 4.6 Spot assays in M9-glucose medium with inosine for purine and thiamin biosynthesis mutants of *E. coli*. Spot assays for *E. coli* purine and thiamin biosynthesis mutants grown next to each other on M9 minimal medium with inosine (50 µM) supplementation, showing results at the end of every 24 h in the P2 passage. Abbreviations: C - *ΔthiC*; K - *ΔpurK*; G - *ΔthiG*; E - *ΔthiE*. Growth of the *ΔpurK* mutant with (A) *ΔthiC*, (B) *ΔthiG*, and (C) *ΔthiE*.

4.3.5 Specific pairs of purine and thiamin biosynthesis mutants survive in co-cultures

We further co-cultured *ΔpurK* and *ΔpurM* mutants with the three thiamin biosynthesis mutants in liquid M9-glucose medium. In the P2 passage, we observed that the co-culture of *ΔthiC*

and *ΔpurK* mutants (termed as the *CK* co-culture) survives in the absence of any externally supplemented inosine and thiamin (Figure 4.7A). On the other hand, co-cultures of *ΔthiE* or *ΔthiG* mutants with the *ΔpurK* mutant (*EK* and *GK* co-cultures, respectively) showed no growth in the absence of external inosine and thiamin supplementation (Figures 4.7B and 4.7C). The *CK* co-culture can grow in unsupplemented M9 medium only if HMP is supplemented by the *ΔpurK* strain to the *ΔthiC* strain, which can salvage it to synthesize thiamin for its own growth, and supplements the *ΔpurK* strain in return with either inosine or some other purine intermediate. However, it seems that the *EK* and *GK* co-cultures do not survive as thiamin is not synthesized in enough amounts by the *ΔpurK* mutant. These results are in line with our hypothesis that as NCAIR biosynthesis does not occur in the *ΔpurK* mutant, it leads to the accumulation of HMP in *ΔpurK*, which can be supplemented to the *ΔthiC* mutant. But thiamin is most likely not supplemented by the *ΔpurK* mutant as THZ biosynthesis in it is not upregulated to the same extent as that of HMP.

We also observed that none of the three co-cultures of the *ΔpurM* mutant with either the *ΔthiC*, the *ΔthiE* or the *ΔthiG* mutant (*CM*, *EM*, and *GM* co-cultures, respectively) survived in the M9 medium in the absence of any inosine and thiamin supplementation (Figures 4.7D-F). This is probably owing to the inefficiency of the *ΔpurM* mutant for the biosynthesis of HMP and, subsequently, that of thiamin (Figure 4.1A). Thus, for *EM* and *GM* co-cultures to survive, *ΔthiE* and *ΔthiG* mutants have additional stress of supplementing two nutrients, namely a purine and HMP, to the *ΔpurM* mutant as explained in hypotheses (iv) and (v) in Figure 4.2. Such a transfer of two or more nutrients in a nutrient-deficient condition when the other strain has only a secondary role in exchange might be more difficult to execute.

On the other hand, *GM* and *EM* co-cultures survive in M9 medium supplemented with inosine, whereas the *CM* co-culture does not (Figure 4.8, data from Vibhor Gaikwad – MS Thesis). This is probably because, the *ΔpurM* mutant phenocopies the *ΔthiC* mutant in the presence of inosine, as it only requires either HMP or thiamin to grow. Thus, in these co-cultures, probably both *ΔthiG* and *ΔthiE* mutants can supplement the *ΔpurM* mutant with HMP, which supplements them back with thiamin, whereas the *ΔthiC* mutant cannot, thus explaining the observed growth phenotypes of these three co-cultures.

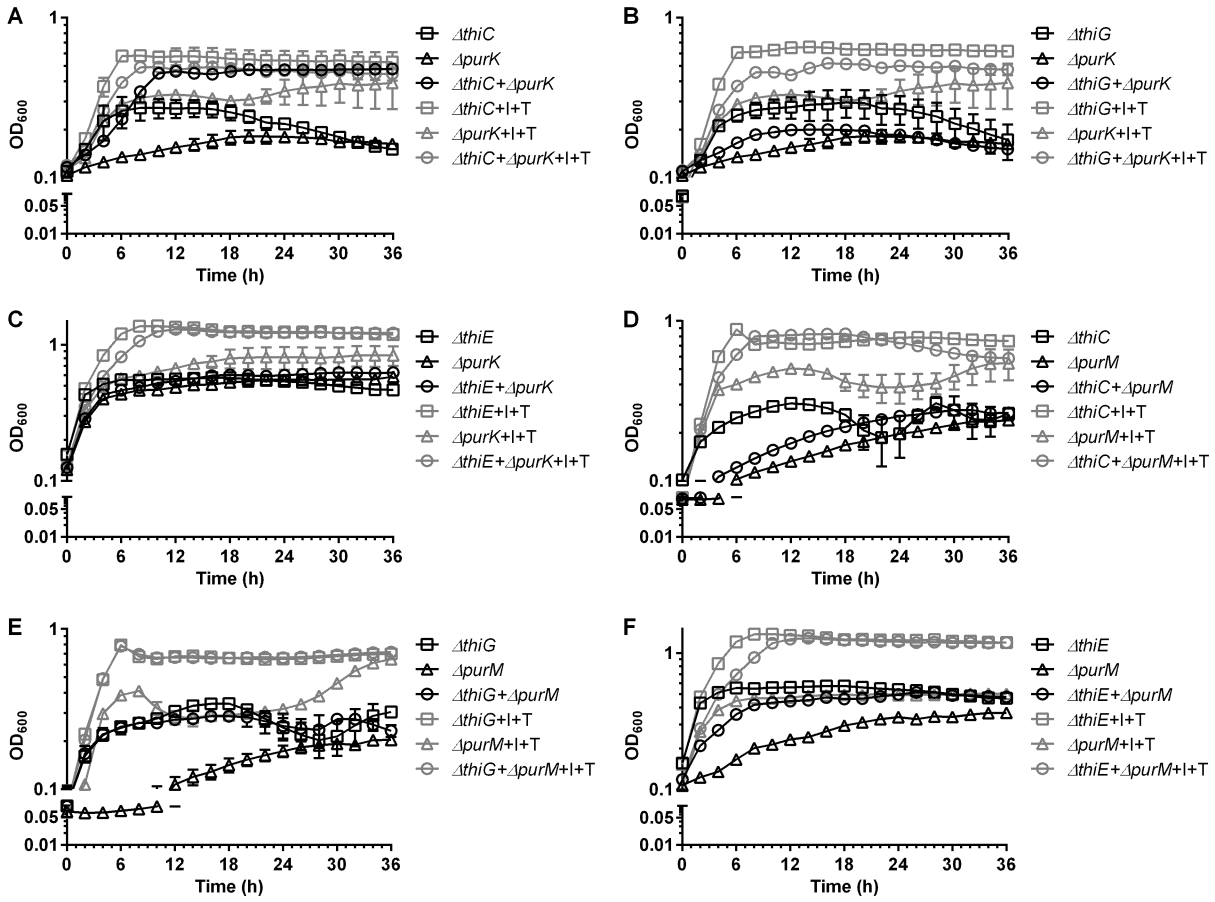

Figure 4.7 Co-cultures of purine and thiamin biosynthesis mutants of *E. coli*. Growth of monocultures and co-cultures of *E. coli* purine and thiamin biosynthesis mutants in the P2 passage of M9 minimal medium. Grey = without supplementation and black = with both inosine (50 µM) and thiamin (20 µM). Co-cultures of the *ΔpurK* mutant with (A) *ΔthiC*, (B) *ΔthiG*, and (C) *ΔthiE* and those of the *ΔpurM* mutant with (D) *ΔthiC*, (E) *ΔthiG*, and (F) *ΔthiE* strains. Means ± standard errors of the means of results from three independent experiments are plotted.

Taken together, we observed that only specific pairs of purine and thiamin biosynthesis mutants of *E. coli* survive in the absence of exogenous supply of either thiamin or both inosine and thiamin. The results obtained for the growth of these co-cultures in M9-glucose media with are enlisted in Table 4.2.

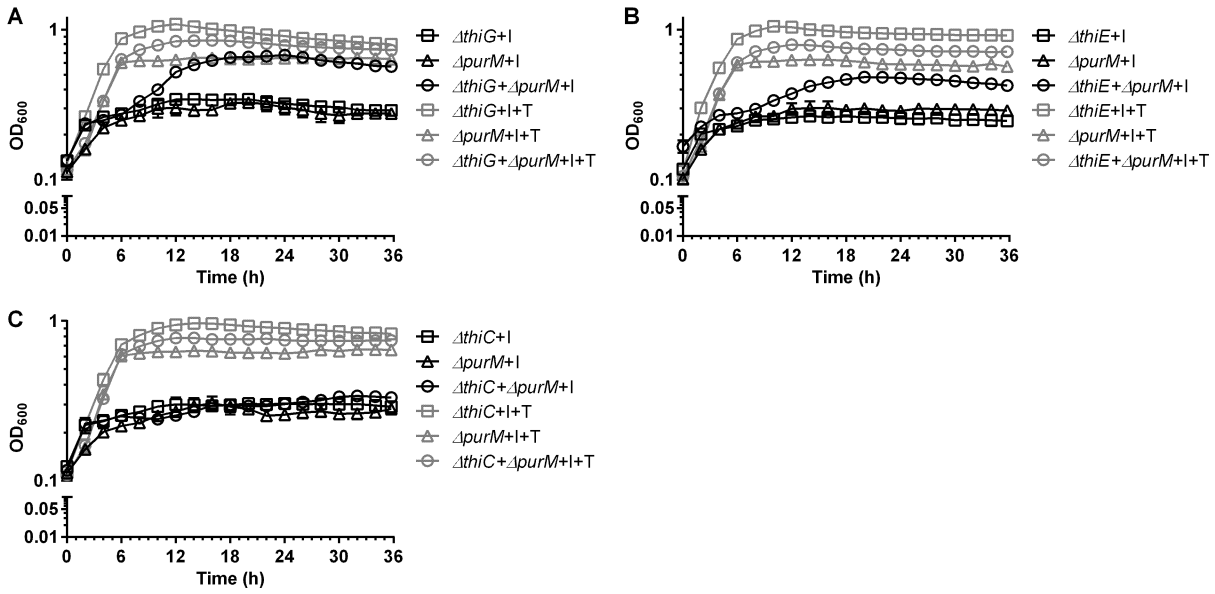

Figure 4.8 Co-cultures of the *ΔpurM* mutant with thiamin mutants of *E. coli* in M9 medium with inosine. Co-cultures of the *ΔpurM* mutant with (A) *ΔthiG*, (B) *ΔthiE*, and (C) *ΔthiC* strains. Means ± standard errors of the means of results from three independent experiments are plotted. Grey = with inosine (25 µM) and black = with both inosine (25 µM) and thiamin (20 µM). Data from Vibhor Gaikwad – MS Thesis.

Co-culture	Growth in M9-glucose medium	Growth in M9-glucose medium with inosine
CK	Yes	N/A
EK	No	N/A
GK	No	N/A
CM	No	No
EM	No	Yes
GM	No	Yes

Table 4.2 Growth of co-cultures of purine and thiamin biosynthesis mutants of *E. coli* in different liquid M9-glucose media. N/A – experiment not performed as the *ΔpurK* strain can survive alone in M9-glucose medium with inosine. Magenta = growth, blue = no growth.

4.3.6 Co-culture of the ΔthiC mutant with the ΔpurK ΔthiG double mutant grows in the M9-glucose medium in the absence of any external supplements

We next cultured the *ΔthiC* mutant with the *ΔpurK ΔthiG* double mutant to validate the exchange of HMP. As the *ΔpurK ΔthiG* double mutant is defective for the synthesis of both inosine and thiazole, even though it has accumulated HMP, it cannot synthesize thiamin. Thus, this co-culture can grow in M9 medium without supplements only if HMP is provided by the *ΔpurK ΔthiG* mutant to the *ΔthiC* mutant, which in return, provides the *ΔpurK ΔthiG* mutant with either inosine or a purine and thiamin (Figure 4.9A). We indeed observed that a co-culture of these two mutants survives in the M9 medium without supplementation of inosine and thiamin (Figure 4.9B).

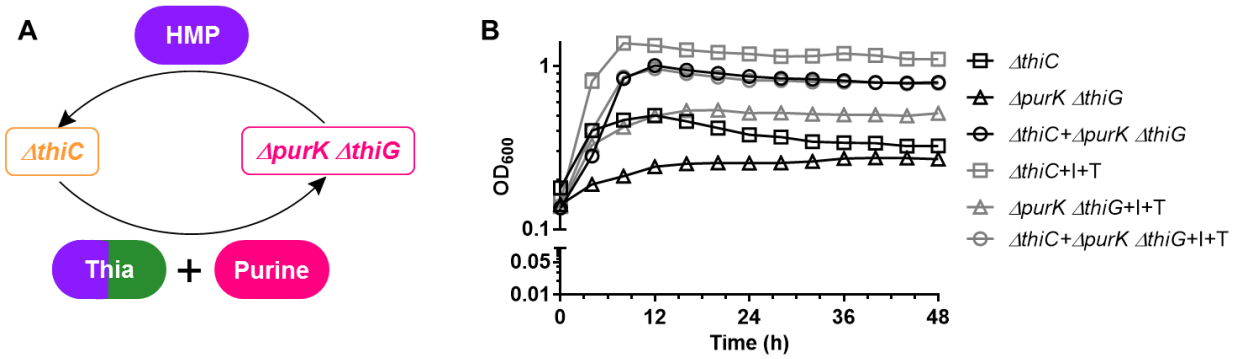

Figure 4.9 Exchange of intermediates in the co-culture of *ΔthiC* and *ΔpurK ΔthiG* mutants of *E. coli*. (A) Model depicting the probable exchange between and (B) co-culture of *ΔthiC* and *ΔpurK ΔthiG* in M9 medium in the P2 passage. T/ Thia = thiamin, I = inosine. Grey = without supplementation and black = with both inosine (50 µM) and thiamin (20 µM). Means ± standard errors of the means of results from three independent experiments are plotted.

We also cultured the *ΔthiG* mutant with the *ΔpurK ΔthiC* double mutant. Because even though AIR accumulates in the *ΔpurK ΔthiC* mutant, it cannot synthesize HMP due to lack of the *thiC* gene. Thus, one of the ways this co-culture could grow in M9 medium without supplements is if THZ is provided by the *ΔpurK ΔthiC* mutant to the *ΔthiG* mutant, which in return, provides

both thiamin and either inosine or a purine to the *ΔpurK ΔthiC* mutant. But as THZ is not exchanged in co-cultures of *E. coli*, we hypothesized that such an exchange of nutrients might not take place[9]. Alternatively, this co-culture could survive if it behaves similar to a *CG* co-culture, wherein the *ΔthiG* mutant supplements the *ΔpurK ΔthiC* mutant with HMP and either inosine or a purine, which in turn synthesizes thiamin and supplements it back to the *ΔthiG* mutant, as depicted in Figure 4.10A. But we rather observed that this co-culture fails to grow in the M9 medium without supplementation of both inosine and thiamin (Figure 4.10B).

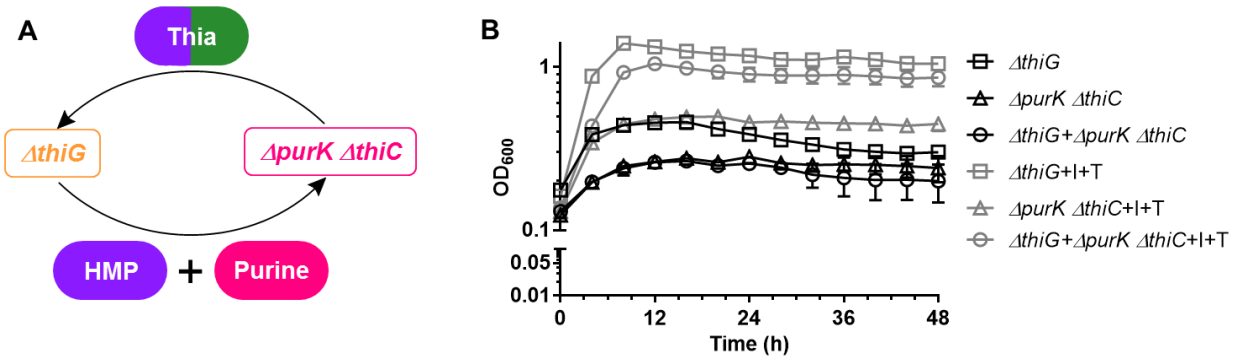

Figure 4.10 Exchange of intermediates in the co-culture of *ΔthiG* and *ΔpurK ΔthiC* mutants of *E. coli*. (A) Model depicting the probable exchange between and (B) co-culture of *ΔthiG* and *ΔpurK ΔthiC* in M9 medium in the P2 passage. T/ Thia = thiamin, I = inosine. Grey = without supplementation and black = with both inosine (50 µM) and thiamin (20 µM). Means ± standard errors of the means of results from three independent experiments are plotted.

4.3.7 Calculating the ratio of strains in the CK co-culture

We next calculated the ratio of individual strains in the *CK* co-culture in P2 passage in the presence and absence of inosine and thiamin. We observed that the ratio of mutants in the *CK* co-culture changes over time irrespective of inosine and thiamin supplementation (Figure 4.11). The percentage of the *ΔthiC* strain in the co-culture increases to about 40%-50% till around 8h-10h, which later drops down to the initial value of about 10% (1:9 starting ratio of the *ΔthiC* strain to

the *ΔpurK* strain). The exact dynamics of this interaction is yet unclear to us, and would need more replicates and variations of the experiment. The results obtained might be due to either (i) a competition for other nutrients among these two mutants once they have gathered enough purine and thiamin for their survival or (ii) lysis of the *ΔthiC* strain to provide inosine to the *ΔpurK* strain.

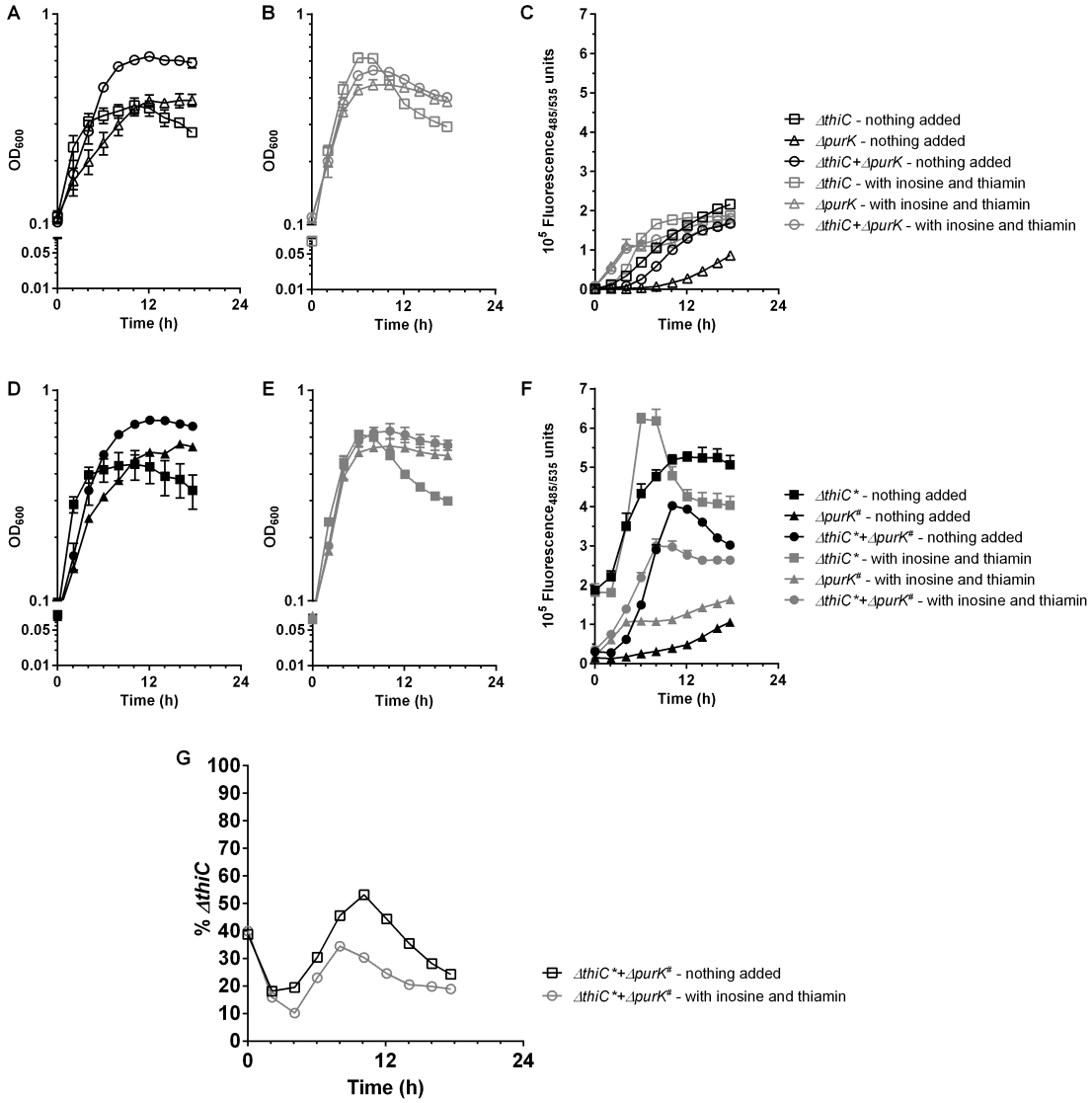

Figure 4.11 Ratio of two strains over time in co-cultures of purine and thiamin biosynthesis mutants of *E. coli* in P2 passage of M9-glucose medium. (A) and (B) OD_{600} and (C) fluorescence at 535 nm of the *CK* co-culture. (D) and (E) OD_{600} and (F) fluorescence at 535 nm of the $C^*K^\#$ co-

culture. (G) Percentage of the *ΔthiC* strain. Black – nothing added, gray – with inosine and thiamin. Means ± standard errors of the means of results from three independent experiments are plotted.

4.4 Summary

Analysis of the exchange of intermediates among purine and thiamin biosynthesis mutants of *E. coli* showed that HMP is the prominently exchanged intermediate of the thiamin biosynthesis pathway in *E. coli*. Among all co-cultures, only the *CK* co-culture could survive in the absence of inosine and thiamin in the minimal medium due to exchange of HMP and a purine intermediate. Also, *EM* and *GM* co-cultures could survive with inosine supplementation in the minimal medium due to exchange of HMP and thiamin. Prominent exchanges of such intermediates on biosynthesis pathways of B vitamins have been reported to shape interactions among various microbes[10–12].

Exchange of purines such as adenine, guanine, hypoxanthine, and xanthine has also been widely reported in literature[13–16]. Synthetic co-cultures of *Saccharomyces cerevisiae* mutants have been shown to exchange adenine and lysine[13]. In yet another study, *Bacillus megaterium* has been shown to lyse and provide purines such as adenine, guanine, hypoxanthine and xanthine to *Ketogulonicigenium vulgare*, thus helping to improve its production of 2-ketogulonic acid, a precursor for vitamin C, on an industrial scale[14]. Additionally, it has been shown that *E. coli* and *Neurospora crassa* fed with either adenine or adenosine excrete hypoxanthine and xanthine in the medium[17,18]. Also, factors affecting the purine biosynthesis pathway such as formate incubation or absence of hypoxanthine-guanine phosphoribosyltransferase (HPT) and adenine kinase (AK) increase excretion of hypoxanthine and xanthine by mammalian cell lines[19,20]. All these studies support our hypothesis of a purine intermediate being exchanged in the *CK* co-culture of *E. coli*.

On the contrary, *EM* and *GM* co-cultures could not survive in the absence of inosine and thiamin in the minimal medium. This might highlight the difficulty in providing two or more nutrients by the primary donor to the secondary donor, which returns a single nutrient. Incidentally, this might mean that the primary donor mostly supplies an intermediate, while the secondary donor supplies an end-product of a pathway, thus leading to such a phenomenon. Apart from this, the PurR regulon of *E. coli* upregulates *thiC* and *thiE* genes by 6.96- and 5.91-fold, respectively, in

the presence of adenine via the PurR-hypoxanthine complex[21]. Thus, adding inosine to the medium might also help increase the cross-talk among purine and thiamin biosynthesis mutants of *E. coli*.

Additionally, *ΔthiC* and *ΔpurK* mutants failed to grow on solid M9 minimal medium without added inosine and thiamin when spotted next to each other, whereas their co-culture in the liquid M9 medium could survive in the absence of these supplements. But the *ΔpurK* strain growing on M9 agar with inosine supported the growth of the *ΔthiC* strain by supplementing it with HMP. Thus, it is likely that either inosine or the purine intermediate supplied by the *ΔthiC* strain to the *ΔpurK* strain is not be available in enough quantities such that it can diffuse through the solid medium, but becomes available in a well-mixed environment in the liquid medium.

The ratio of mutants in the *CK* co-culture also fluctuates irrespective of the supplementation with inosine and thiamin in the minimal medium, a phenomenon that would require further investigation. This might point to either a competition among the two strains for other nutrients in the medium, or to the lysis of the *ΔthiC* strain. Such exchange of nutrients shaped by competition has been shown to be prominent than cooperation in microbial communities[22–24].

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Chapter 5: Visualizing the growth patterns formed due to cross-talk among thiamin biosynthesis mutants of *Escherichia coli* K-12 MG1655

5.1 Spatial patterns formed due to cross-talk are affected by a variety of factors

Microbes that are involved in a nutrient-based cross-talk often arrange themselves in a spatiotemporal manner, such as those in cheese rinds, animal gut, root nodules, and even those in the form of disease-causing biofilms[1–6]. Synthetic microbial cultures have also been analyzed to study the ecological and evolutionary phenomena affecting the spatial arrangement of organisms[7–9]. For example, a synthetic symbiotic community of three microbes – *Azotobacter vinelandii*, *Bacillus licheniformis*, and *Paenibacillus curdlanolyticus* was shown to survive only when the three were separated by an optimal distance from each other, but not in mixed cultures or monocultures separated by a large distance[10]. Semi-natural communities of pollutant degrading microorganisms and nitrogen-fixing bacteria were also shown to benefit from spatial patterning due to the nature of biochemical reactions[11,12]. Mutants in a synthetic community of two *Saccharomyces cerevisiae* mutants engaged in the exchange of adenine and lysine have also been shown to arrange themselves in different patterns[13]. Thus, we visualized the exchange of nutrients among thiamin biosynthesis mutants of *E. coli* K-12 MG1655 by analyzing the growth of colonies of co-cultures of thiamin mutants using microscopy.

We first checked the growth of *CE* and *CG* co-cultures of *E. coli* in the presence and absence of thiamin to determine the effect of its availability on spatial patterns formed by these strains. We also studied the spatial arrangement of these three *thi*$^-$ mutants in a three-membered community. Further, we calculated the ratio of mutants in these two- and three-membered cultures.

We observed that in the absence of thiamin, two- and three-membered co-cultures of *thi*$^-$ mutants showed co-localization, which was not observed in the presence of thiamin. This showed that these mutants engaged in an active cross-talk in the absence of exogenous thiamin, and thus interacted and co-localized with each other. The ratios of mutants in two- and three-membered co-cultures of *thi*$^-$ mutants also changed in the absence of thiamin, indicative of a cross-talk.

5.2 Materials and Methods

5.2.1 Chemicals and reagents: Inosine was obtained from TCI chemicals and thiamin and sodium fumarate were obtained from Sigma-Aldrich. All other chemicals, reagents and kits used were obtained either from TCI chemicals, HiMedia or Sigma unless otherwise specified.

5.2.2 Strains and plasmids: *E. coli* K-12 MG1655 strains *ΔthiC*, *ΔthiE*, *ΔthiG*, *ΔthiC**, *ΔthiE**, and *ΔthiG** (* denotes the presence of the *GFPmut2-kan*^{R} cassette) were constructed in this study (Chapters 2 and 3). *E. coli* K-12 MG1655 *ΔthiC*, *ΔthiE*, and *ΔthiG* mutants were made chemically competent and transformed by Vibhor Gaikwad (MS Thesis – Vibhor Gaikwad) with pMRE145 and pMRE141 plasmids from Addgene containing the mScarlet and Turquoise fluorescent genes, respectively, (termed, respectively, for example, for the *ΔthiC* mutant, as *ΔthiC*$^{\#}$ and *ΔthiC*$^{\$}$).

5.2.3 Growth conditions and media:

5.2.3.1 Primary culture set-up (LB and P1 cultures): LB and P1 cultures of all strains were set up in the same manner as previously described (Chapter 2, Materials and Methods, Section 2.2.4.1).

5.2.3.2 Secondary colony spots set-up (P2 cultures): Cells washed after the P1 passage were used for colony spot assays as their P2 cultures on M9-fumarate [M9 + NH_4Cl + 0.2% Na-fumarate] agarose plates, with 1.5% w/v agarose, supplemented with or without thiamine (20 μM). All different strains were normalized to an OD_{600} of 0.1, which is considered to be the culture density of ~10^7 cells per mL for *E. coli* K-12 MG1655. For this, cultures were spotted as 2 μL spots on the above-mentioned plates at a starting OD_{600} of 0.1, either directly as monocultures or as co-cultures (mixed in an eppendorf using 1X M9 salts, in 1:9 ratio, in the same way for two-membered co-cultures as mentioned in Chapter 3, Section 3.2.3.2) to start their growth as P2 cultures, and were incubated aerobically at 37°C, for 3 days. For the three-membered community assays, cells were mixed at a starting ratio of 1:4.5:4.5 of *ΔthiC*:*ΔthiE*:*ΔthiG* strains.

5.2.3.3 Colony forming unit (CFU) count: Cells grown on solid M9-fumarate medium with or without thiamin (20 μM) were used for the CFU count experiment. After growth for 3 days as P2 cultures, colonies formed were scooped out with a sterile spatula along with the agar and re-suspended in 10 mL of 1X M9 salts by vortexing for 30 sec in a 50 mL falcon. From this, 30 μL of re-suspended cells were transferred to 270 μL of 1X M9 salts (10-fold dilution), and

this step was repeated for further 10-fold dilutions for 6 times in a 96-well plate. Post this, 10 µL of cells from all 7 dilutions were spotted on one corner of a single LB plate with Kanamycin (25 µg/ mL), the plate was tilted to let the spot drag, run, and dry-out, and was then incubated at 37°C overnight. The next day, CFUs were counted for cells by imaging them under GFP and mCherry (fluorescent) channels and the visible channel in SynGene G:box to get an estimate of the ratio of different strains in the co-culture.

5.2.4 Microscopy analysis: Colonies formed by monoculture and co-culture spots on agar plates were analyzed under a microscope after every 24 h. For initial standardization of media conditions and assays, plates were imaged under a Zeiss Visitron system, 2.5X magnification objective and Photometrics PRImE 95B camera, using following filter and exposure conditions: bright-field for 10 ms at 48% intensity and GFP (470/20 nm, 525/25 nm) for 10 ms at 30%, at Dr. Sara Mitri's lab, DMF, University of Lausanne, Switzerland. Experiments included in this chapter have been carried out using a Leica DM6 system, HC PL FLUOTAR 5x/0.15 DRY objective and Hamamatsu C11440-22CU camera, using following filter and exposure conditions: bright-field for 100 ms at 40% intensity, DsR (546/6 nm, 605/38 nm) for 20 ms at 30% intensity, SBL (405/5 nm, 460/20 nm) for 2000 ms at 30% intensity, and GFP (470/20 nm, 525/25 nm) for 100 ms at 30% intensity. Images obtained were processed using the Fiji software for converting stacks to images, labelling channels and adding scale bars[14].

5.3 Research design, Results, and Discussion

5.3.1 Patterns observed for CE and CG co-cultures in the presence and absence of thiamin

CE and *CG* co-cultures of *E. coli* grown on M9-fumarate agarose medium were analyzed for differences in their spatial arrangement in the presence and absence of thiamin as described earlier. $C^{\#}E^{*}$ and $C^{\#}G^{*}$ co-cultures ($^{\#}$ = scarlet fluorescence, * = green fluorescence) were spotted at a 1:9 starting ratio of the *ΔthiC* mutant to the *ΔthiE* or the *ΔthiG* mutant similar to previous assays. In the presence of thiamin, after 48-72 h of growth, $C^{\#}E^{*}$ and $C^{\#}G^{*}$ co-cultures showed lesser scarlet fluorescence than green fluorescence (Figures 5.1A and 5.2A). This shows that the number of *ΔthiC* cells in the spot is lesser than that of the *ΔthiE* or *ΔthiG* cells, which verifies that the ratio of thi^{-} mutants in a co-culture does not change when supplemented with thiamin. On the contrary, in the absence of thiamin, $C^{\#}E^{*}$ and $C^{\#}G^{*}$ co-cultures showed higher scarlet fluorescence

than green fluorescence (Figures 5.1B and 5.2B). This shows that the number of *ΔthiC* cells in the co-spot is higher than that of *ΔthiE* or *ΔthiG* cells, which verifies that the ratio of *thi*$^{-}$ mutants in a co-culture almost reverses when supplemented with thiamin.

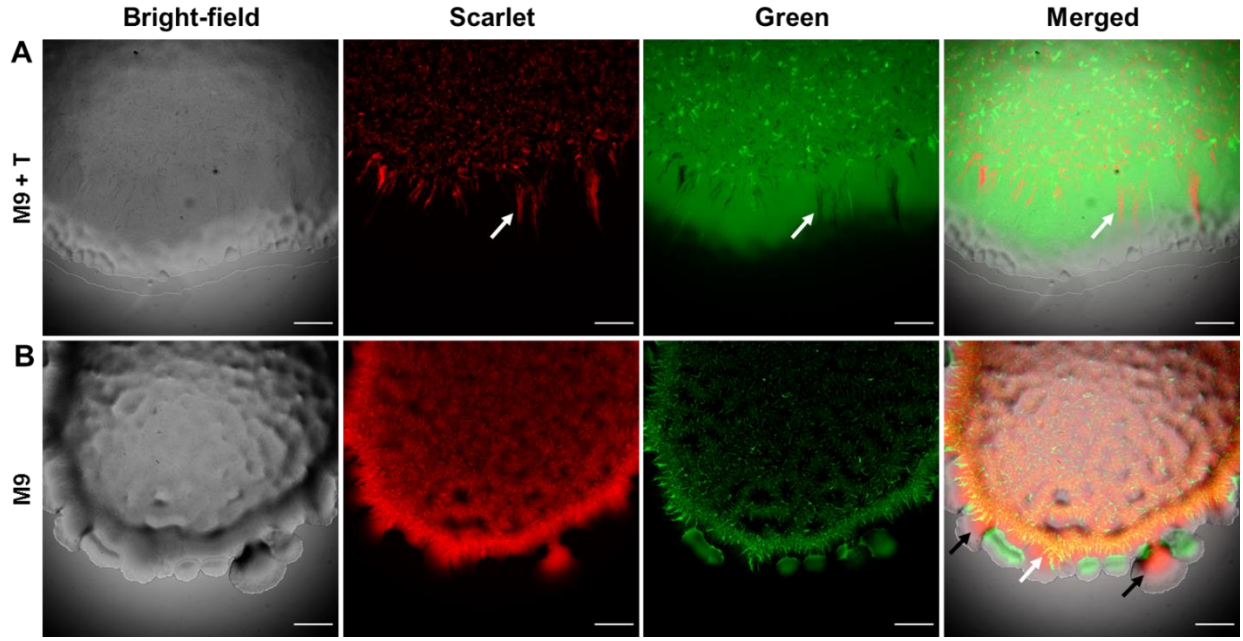

Figure 5.1 Visualizing the *C$^{\#}$G co-culture of *E. coli*.** *C$^{\#}$G** co-culture after 48 h in P2 on M9-fumarate medium: (A) with thiamin and (B) without thiamin. Scale bar = 500 µm. White arrows indicate the non-overlap of two strains in (A) in comparison to the overlap in (B). Black arrow indicates the advancing border of *ΔthiC* cells. $^{\#}$ = scarlet and * = green fluorescence.

Additionally, we observed that in the absence of thiamin, *ΔthiC* cells are present at the advancing border of the colonies, followed by those of the other strain (Figures 5.1B and 5.2B, black arrows). This might indicate that, as the *ΔthiC* cells supplement thiamin, the final molecule made in the pathway, at 48 h, they can survive on their own and hence advance ahead towards nutrient-rich area. But as the *ΔthiE* or *ΔthiG* cells are dependent on thiamin supplied by the *ΔthiC* cells, they follow the latter. We also observed that these images show alternate invaginations of the two strains in the co-culture when thiamin is present, whereas, in the absence of thiamin, more

overlap is seen among the two strains as indicated by the yellow/ orange colour in the images (Figures 5.1 and 5.2, white arrows). This likely indicates the co-dependence of two strains in the absence of thiamin for the nutrients HMP and thiamin.

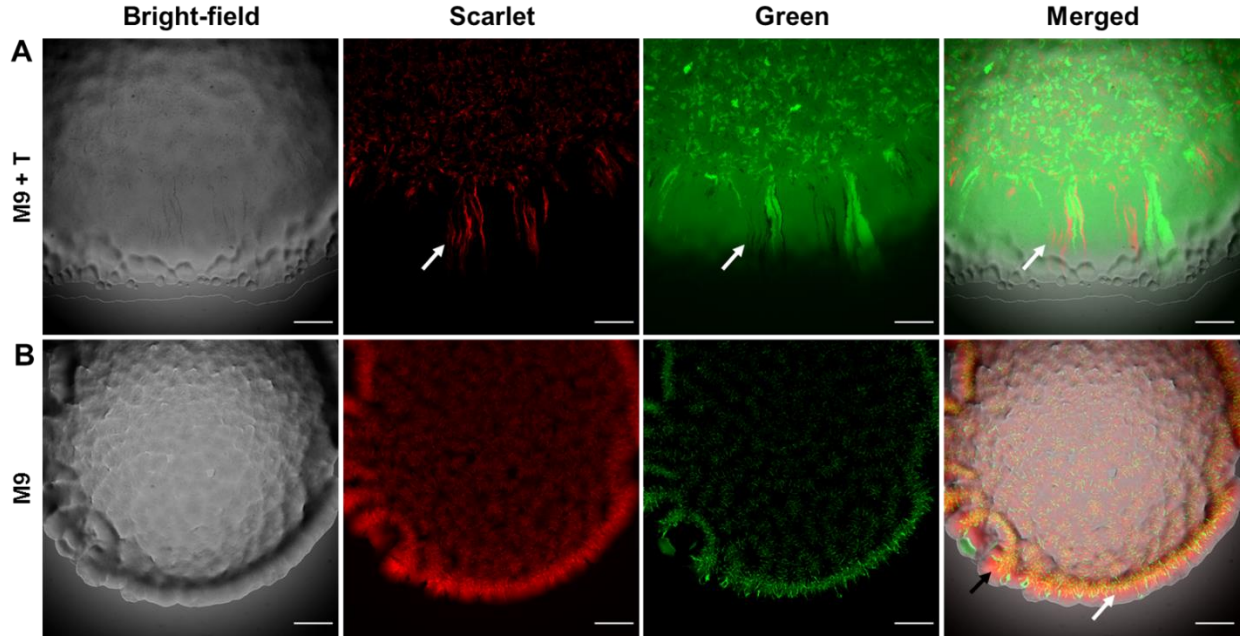

Figure 5.2 Visualizing the *C#E co-culture of *E. coli*.** *C#E** co-culture after 48 h in P2 on M9-fumarate medium: (A) with thiamin and (B) without thiamin. Scale bar = 500 µm. White arrows indicate the non-overlap of two strains in (A) in comparison to the overlap in (B). Black arrow indicates the advancing border of *ΔthiC* cells. # = scarlet and * = green fluorescence.

5.3.2 Patterns observed for a three-membered community of thi⁻ mutants

We next analyzed the pattern observed in a three-membered community of *ΔthiC#*, *ΔthiE**, and *ΔthiG$* strains (# = scarlet fluorescence, * = green fluorescence, $ = turquoise fluorescence, thus termed *C#E*G$* co-culture), started at a 1:4.5:4.5 ratio, in the P2 passage on M9-fumarate medium in the presence and absence of thiamin. On the medium containing thiamin, the three-membered community showed a similar phenotype as the pairwise co-cultures, with the scarlet

fluorescence being lesser (Figures 5.3A and 5.4A – *ΔthiC*$^{\#}$, *ΔthiE*$^{\$}$, and *ΔthiG** strains used in figure 5.4, thus termed *C*$^{\#}$*E*$^{\$}$*G** co-culture). This shows that the number of *ΔthiC* cells in this community is less, indicating that the ratio of strains in this community might not deviate much from the starting ratio. On the contrary, when thiamin was absent, the three-membered community showed higher scarlet fluorescence (Figures 5.3B and 5.4B). This shows that the number of *ΔthiC* cells in this community is high, indicating that the ratio of strains in this community might have changed from the ratio at the start of this culture. We also observed a co-localization of strains (white arrows) in the absence of thiamin (Figures 5.3B and 5.4B).

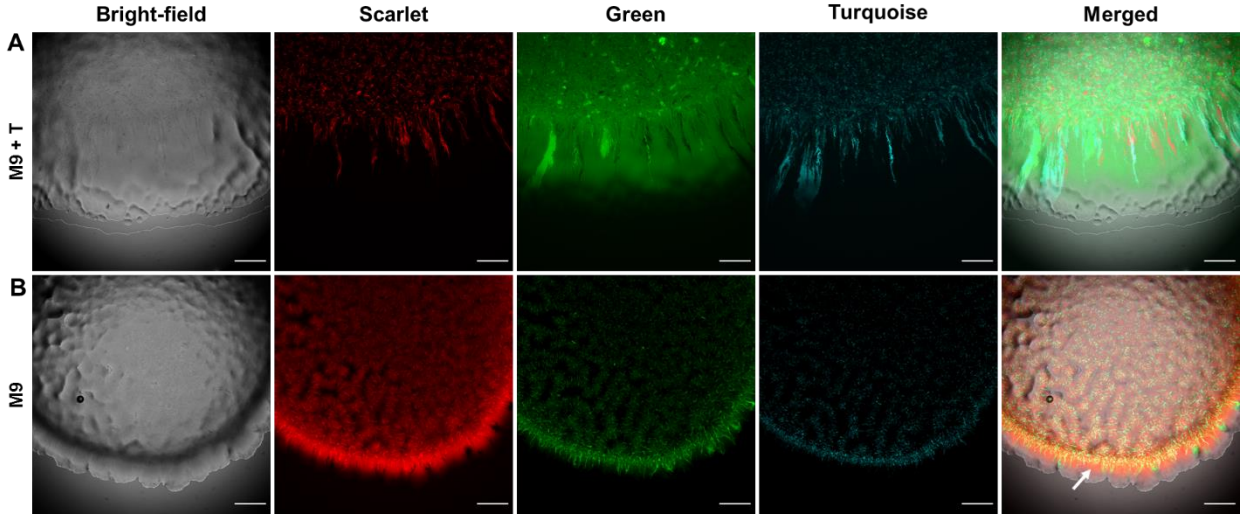

Figure 5.3 Visualizing the *C*$^{\#}$*E*G*$^{\$}$ co-culture of *E. coli*. *C*$^{\#}$*E*G*$^{\$}$ co-culture after 48 h in P2 on M9-fumarate medium: (A) with thiamin and (B) without thiamin. Scale bar = 500 µm. White arrows indicate the overlap of strains in (B). $^{\#}$ = scarlet, * = green, and $^{\$}$ = turquoise fluorescence.

Curiously enough, the turquoise fluorescence in figures 5.3A and 5.4A seems to be lower than the green fluorescence, irrespective of the *thi*$^{-}$ mutant tagged with it. Also, the overall growth of turquoise mutants seemed to be lesser than scarlet or GFP mutants, indicating that the turquoise strain might have some disadvantage in the presence of thiamin, seemingly making the apparent growth of the GFP strain better. Such a difference was not observed in figures 5.3B and 5.4B,

indicating that the fluorescent tag does not give a disadvantage to strains in the absence of thiamin. To address this issue, we used untagged strains in place of turquoise strains, but indeed observed a similarly poor growth of the untagged strain when grown in the presence of thiamin, irrespective of the nature of the untagged strain (Figure 5.5 – *C*$^{\#}$*E*G* and *C*$^{\#}$*EG** co-cultures).

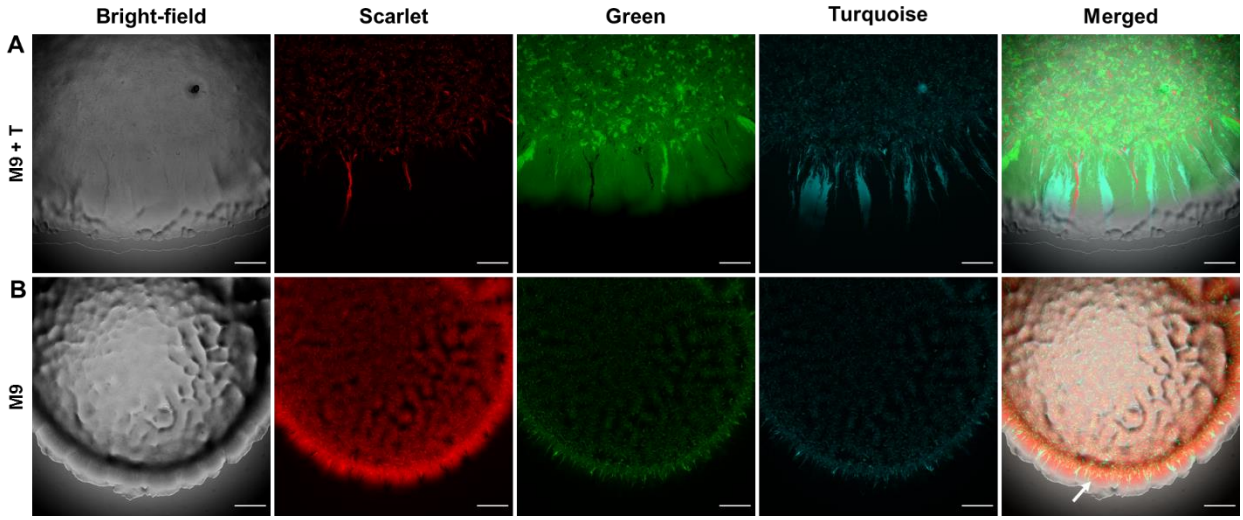

Figure 5.4 Visualizing the *C*$^{\#}$*E*$^{\$}$*G co-culture of *E. coli*.** *C*$^{\#}$*E*$^{\$}$*G** co-culture after 48 h in P2 on M9-fumarate medium: (A) with thiamin and (B) without thiamin. Scale bar = 500 µm. White arrows indicate the overlap of strains in (B). $^{\#}$ = scarlet, * = green, and $^{\$}$ = turquoise fluorescence.

5.3.3 Quantitation of ratios of thi^{-} mutants in two- and three-membered co-cultures

We further analyzed the percentage of *thi*$^{-}$ mutants in *C*$^{\#}$*E**, *C*$^{\#}$*G**, *C*$^{\#}$*E*G* and *C*$^{\#}$*EG** co-cultures in the presence and absence of thiamin using the CFU-based quantitation assay as described earlier. In case of *C*$^{\#}$*E** and *C*$^{\#}$*G** co-cultures, results obtained were similar to those in Chapter 3, with the ratio not varying from the starting ratio in the presence of thiamin, and the percentage of the *ΔthiC* strain increasing in its absence (Figure 5.6).

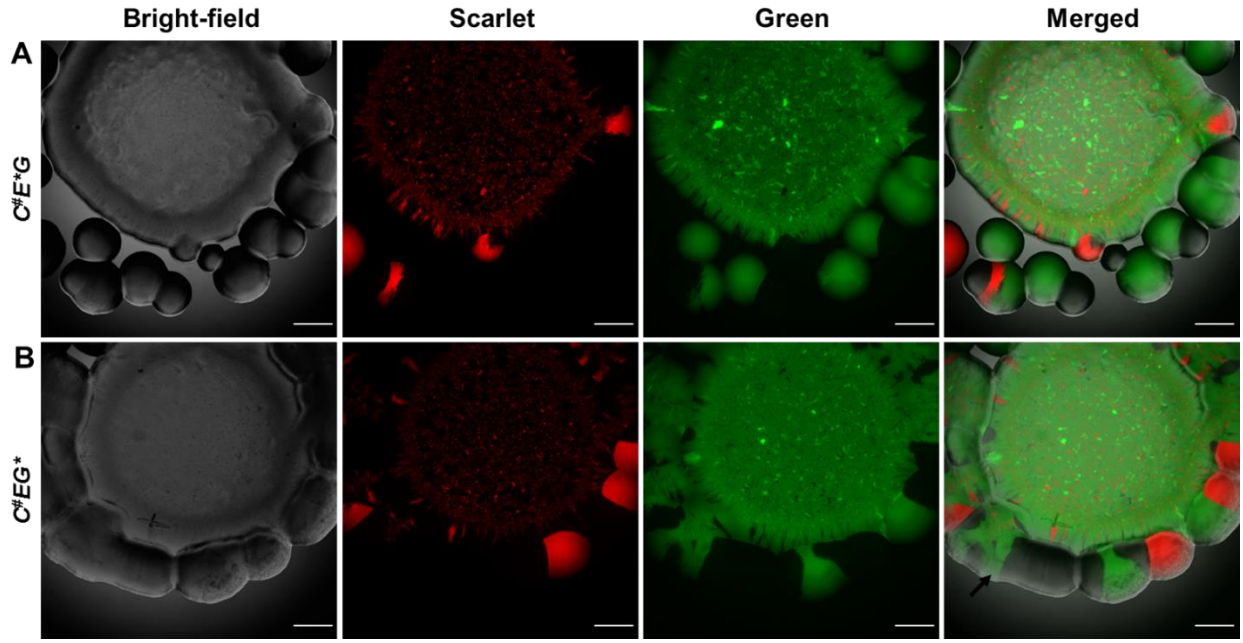

Figure 5.5 Visualizing co-cultures of *E. coli*. (A) *C#E*G* and (B) *C#EG** co-cultures at 72 h, on M9-fumarate medium with thiamin. Scale bar = 500 µm. # = scarlet and * = green fluorescence.

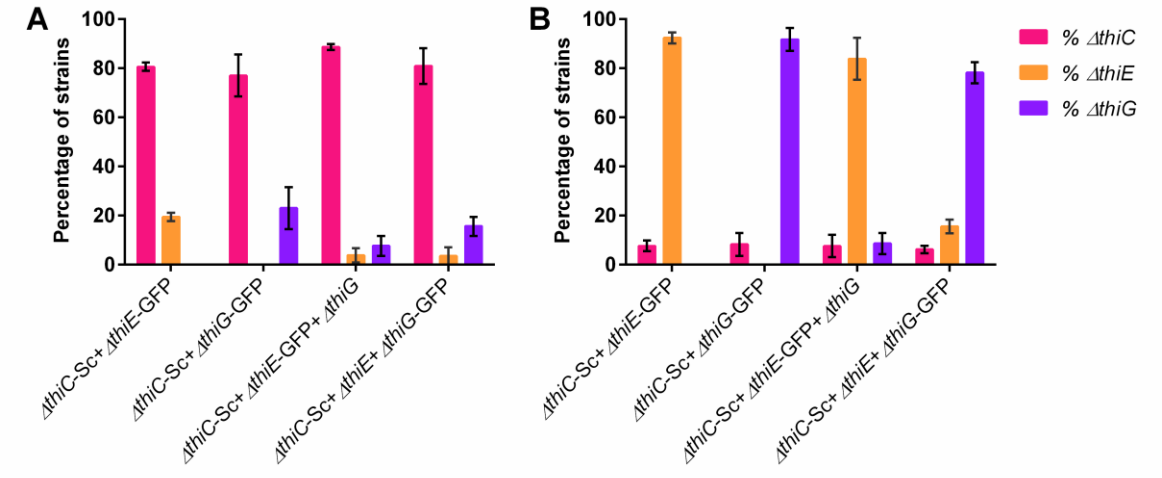

Figure 5.6 Quantitating percentage of strains in co-cultures of *E. coli*. Percentage of strains in two- and three-membered co-cultures of *E. coli* after 72 h of growth in P2 on M9-fumarate medium: (A) without thiamin and (B) with thiamin. Sc = scarlet and GFP = green fluorescence.

In the absence of thiamin, we indeed observed that similar to pairwise co-cultures, the percentage of the *ΔthiC* strain in the three-membered co-cultures $C^{\#}E^{*}G$ and $C^{\#}EG^{*}$ also increased to ~80%, whereas the rest ~20% was made up by other strains in the co-culture (Figure 5.6A). In the presence of thiamin, the percentage of the *ΔthiC* strain did not deviate much from the starting value, but that of the GFP-tagged strain was higher than the untagged strain, for both $C^{\#}E^{*}G$ and $C^{\#}EG^{*}$ co-cultures, similar to the phenomenon observed for the microscopy images, and requires further experiments (Figure 5.6B).

5.4 Summary

Visualization of two- and three-membered co-cultures of thiamin mutants under a microscope showed lesser co-localization for them when thiamin is present in the medium. But in the absence of thiamin, both two- and three-membered co-cultures of thiamin mutants showed higher co-localization. This shows that in the presence of nutrients, these mutants do not face the need to interact with each other and rather grow independently by utilizing and competing for the nutrients provided by the medium and the total space available, a phenomenon observed earlier for microbes[15]. On the other hand, in the absence of the growth-limiting nutrient thiamin, these mutants seem to engage in a cooperative cross-talk by exchanging intermediates on thiamin biosynthesis pathway, and hence co-localize with each other, likely facilitating the exchange. Such co-localization might be passive in nature, as the mutants are co-spotted and hence likely occupy the same niche at any given point. Thus, with an increase in the local concentration of one nutrient, the mutant that can use it proliferates at that place.

We also observed that the percentage of the *ΔthiC* strain in both two- and three-membered communities increases to ~80% in the absence of thiamin. This corroborates the result obtained by us in Chapter 3, which showed that when thiamin is not present in the medium, the *ΔthiC* strain is able to make thiamin by utilizing the HMP provided by *ΔthiE* or *ΔthiG* strains in pairwise co-cultures and hence proliferates better than the latter. It is likely that even in case of the three-membered community, in the absence of thiamin, the *ΔthiC* strain is able to provide enough thiamin to two other strains in return for a constant, yet low supply of HMP provided by their small number of cells. It is also interesting to note that in both the cases, where the *ΔthiC* strain has to support either one or two strains, the total percentage of strains supported is ~20%, indicating that

the thiamin synthesizing strain can only support a population 1/4th of its own population size. Such cooperation based intermixing of strains, with their ratios converging to a particular number has been observed earlier for yeast mutants[15]. Changes in ratios of microorganisms involved in the exchange of thiamin and other nutrients have also been reported earlier in literature[16–18].

In the presence of thiamin, for pairwise co-cultures, we observed that the ratio of strains in the co-culture does not deviate much from the starting ratio, similar to the results obtained in Chapter 3. This further verifies that the two strains do not interact with each other when thiamin is available in the medium. But in the three-membered community, the GFP-tagged strain grew better than the turquoise-tagged or untagged strain in the presence of thiamin, irrespective of the strain tagged with GFP. As the GFP tagged strains used here are the same as those in chapter 3 where such tag-based differences in growth were not observed, this phenomenon would need further exploration.

5.5 References

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Chapter 6: Other physiological explorations in *Escherichia coli*

6.1 Intricate and complex pathways and similar enzymatic roles

6.1.1 Analyzing the role of ThiS

The thiamin biosynthesis pathway in *E. coli* K-12 MG1655 consists of both *de novo* and salvage enzymes (Chapter 1, Figure 1.4). ThiD, ThiM, ThiK, and ThiL act as kinases which add phosphate groups to 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) and 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P), 4-methyl-5-(2-hydroxyethyl)thiazole (THZ), thiamin, and thiamin monophosphate, respectively[1–3]. Apart from this, a cascade of enzymes are involved in the transfer of sulfur in the thiazole moiety[4]. Dehydroglycine moiety used in the biosynthesis of sulfur is synthesized by yet another enzyme ThiH[5].

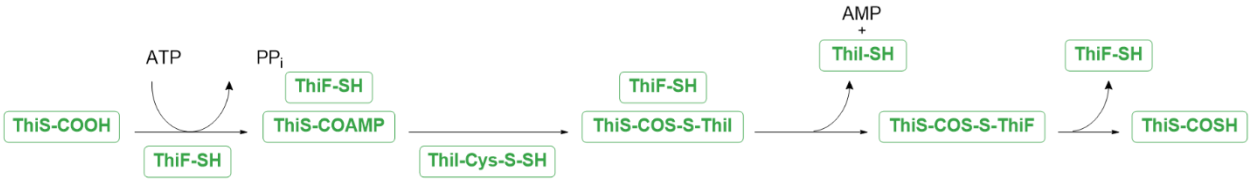

Figure 6.1 Formation of ThiS-thiocarboxylate (ThiS-COSH) in *E. coli*. ThiS-COSH in *E. coli* is formed via a relay of sulfur-transfer reactions, using multiple enzymes.

An interesting feature of thiamin biosynthesis in *E. coli* is that it involves a small sulfur-carrier protein ThiS, which has its C-terminus post-translationally modified to a thiocarboxyl group that contributes the sulfur in the thiazole ring of thiamin[6]. The prokaryotic enzyme ThiS is not a suicide enzyme unlike the eukaryotic enzyme THI4, which donates the sulfur from its own cysteine to form the thiazole ring[7,8]. Instead, ThiS is first adenylated by the enzyme ThiF, forming a good leaving group which is displaced by the sulfur donated by ThiI (Figure 6.1)[6,9]. ThiI gets this sulfur atom from IscS[10]. Such a relay of sulfur transfer is also observed in several other metabolic pathways, involving those in the biosynthesis of cysteine[11], molybdopterin[12] and

thioquinolobactin[13]. It has also been suggested that sulfur carrier proteins such as ThiS might have led to the origin of ubiquitin[14]. Also, the protein ThiI has been shown to carry a persulfide intermediate that is used to covalently modify different substrates[15]. The sulfur transfers involved in the cofactor and nucleoside biosynthesis are carried out predominantly via the persulfide group (R-S-SH), and the sulfurtransferases usually modify a range of substrates[16]. In order to understand the relay of sulfur in thiamin biosynthesis and overall metabolism in *E. coli*, we created the *ΔthiS* gene knockout mutant of *E. coli* K-12 MG1655, and co-cultured it with *ΔthiC*, *ΔthiE*, and *ΔthiG* mutants (termed as *CS*, *ES* and *GS* co-cultures, respectively).

6.1.2 Analyzing the role of 3-mercaptopyruvate sulfurtransferase (3-MST) in E. coli

Persulfide groups on such enzymes are carried on a rhodanese homology domain, which helps in detoxification of cyanide and alleviating redox stress[17,18]. 3-mercaptopyruvate sulfurtransferase (3-MST) is one of them, which has a rhodanese domain with persulfide group on one of its cysteine residues[19,20]. It uses 3-mercaptopyruvate (3-MP) as a substrate, and generates an enzyme bound-persulfide and pyruvate as a byproduct[21]. The persulfide can then be attacked by a range of substrates, generating H_2S or polysulfides in the process (Figure 6.2)[22]. Persulfides, sulfane sulfur and polysulfides have roles in the cellular metabolism that are greatly unexplored, hence we wish to understand the role of the 3-MST protein in regulating the sulfur-metabolism of *E. coli*[23,24]. This project is in collaboration with Dr. Harinath Chakrapani's lab, IISER Pune, India.

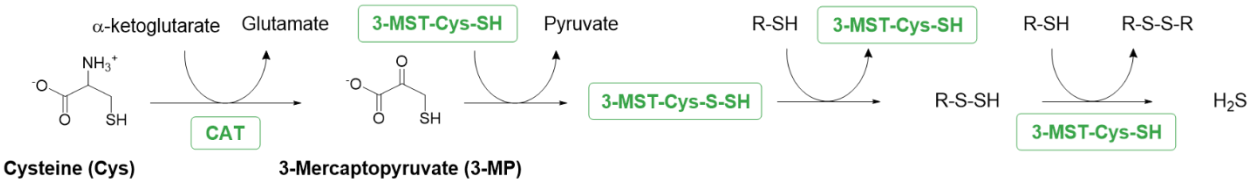

Figure 6.2 Sulfur transfer by 3-MST in *E. coli*. *Ec*-3-MST produces persulfides and polysulfides, generating H_2S in the process.

In addition, H_2S generated by 3-MST has been shown to alleviate oxidative stress and help gain antibiotic resistance to prokaryotes (Figure 6.3)[25,26]. H_2S in eukaryotes also helps in vasorelaxation and alleviating kidney disorders, and thus, is of significant clinical interest[27,28]. H_2S

is known to be produced majorly by three enzymes, cystathionine-β-lyase (CBS), cystathionine-γ-synthase (CSE), and 3-MST in both prokaryotes and eukaryotes, of which, *E. coli* only has 3-MST[28,29]. Apart from these, five other desulfhydrases are known to exist in *E. coli*, but the amount of H_2S produced by them is negligible as compared to that by 3-MST[26]. Knockout mutants of 3-MST with mutations in the *ycjW* gene, a repressor of sulfur transfer proteins such as PspE, have also been found in *E. coli*[30]. 3-MST has an optimum working pH of 8-9 and is localized to mitochondria and cytoplasm[20,31,32]. The reaction mechanism of generation of H_2S by 3-MST has also been proposed based on the crystal structure of human 3-MST[20]. With this knowledge, we also wanted to analyze the antibiotic resistance mechanism of 3-MST in prokaryotes, and develop a range of novel substrates and inhibitors for it.

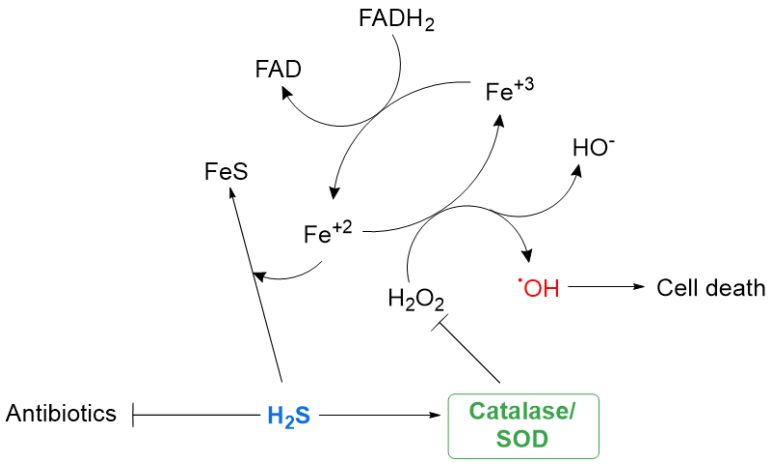

Figure 6.3 Role of H_2S in prokaryotes. H_2S produced inside the cell helps prokaryotes to combat antibiotics by quenching free Fe^{+2} and H_2O_2.

6.1.3 Analyzing the 'yellow compound' produced by the ΔpurK strain

While growing the *ΔpurK* mutant of *E. coli* K-12 MG1655, we also observed a yellow colour in the media in the P1 and P2 passage of M9-glucose medium (Figure 6.4). This was intriguing, as neither the *ΔpurM* mutant nor the wild-type strain produced such a colour in the medium (Figure 6.4). This showed that the yellow colour production is a result of the mutation of

the *purK* gene and is probably related to the accumulation of the purine biosynthesis pathway intermediate 5'-Phosphoribosyl-5-aminoimidazole (AIR), which is produced by PurM and is further committed to the pathway by PurK[33–35]. Literature reports formation of a red-coloured compound by adenine biosynthesis pathway mutants of yeast (*ade2* and *ade1* mutants of *Saccharomyces cerevisiae*, and *ade6* and *ade7* mutants of *Schizosaccharomyces pombe*), which are homologues of *purK* and *purC* genes, respectively, in *E. coli*[36–38]. PurC catalyzes the conversion of 5'-Phosphoribosyl-5-amino-4-imidazolecarboxylate (CAIR), which is a product of PurE, the enzyme after PurK in the purine biosynthesis pathway, to 5'-Phosphoribosyl-4-(N-succinocarboxamide)-5-aminoimidazole (SAICAR)[38,39]. Both AIR and CAIR which would accumulate due to these mutations are not coloured, and the conjugate of AIR with glutathione and some other amino acids has been characterized by these authors to constitute the red-coloured pigment in these yeasts[36,37]. Even *Neurospora crassa* adenine mutants of homologues of these enzymes have been shown to produce a purple-coloured pigment[40]. Thus, we also investigated the nature of this yellow-coloured pigment produced by the *ΔpurK* mutant of *E. coli*.

Figure 6.4 Yellow colour produced by the *ΔpurK* mutant of *E. coli*. The *ΔpurK* mutant of *E. coli* shows production of yellow colour in the P1 passage of M9-glucose medium. WT and *ΔpurM* strains, on the other hand, do not produce yellow colour.

6.2 Materials and Methods

6.2.1 Chemicals and reagents: Inosine was obtained from TCI chemicals and 4-methyl-5-hydroxyethyl thiazole and thiamin were obtained from Sigma-Aldrich. All other chemicals, reagents and kits used were obtained either from TCI chemicals, HiMedia or Sigma unless otherwise specified. The enzymes used for generating knockouts, diagnostic PCRs, and cloning were obtained from TaKaRa.

6.2.2 Strains and plasmids: *E. coli* K-12 MG1655 strains *ΔthiC, ΔthiE,* and *ΔthiG* were constructed in this study (Chapter 2). The *E. coli* K-12 BW25113 strain containing the plasmid pKD46 and the *E. coli* K-12 BW25141 strain containing the plasmid pKD3 were a gift from Dr. Nishad Matange, IISER Pune. *E. coli* K-12 MG1655 strains *ΔthiS::cam*^{R}, *ΔsseA::kan*^{R}, and *ΔycjW::kan*^{R} strains (termed as *ΔthiS, ΔsseA,* and *ΔycjW*) were created using the λ-red recombineering system, and steps for primer design, mutant generation, and verification were carried out as previously described, and were described in detail in Chapter 2, Materials and Methods, Section 2.2.3, and primers used are listed in Table 6.1 in this chapter [41,42]. The wild type MST-encoding gene, *sseA,* from *E. coli* K-12 MG1655 was cloned by Mrutyunjay Nair (MS Thesis – Mrutyunjay Nair) in pET28a vector using restriction-free cloning method as previously described[43]. Using this *Ec*3-MST-pET28a plasmid, a forward primer with C238A mutation and T7 reverse primer, and restriction-free cloning method, C238A mutation was introduced in *Ec*3-MST. *Ec*3-MST-pET28a and C238A *Ec*3-MST-pET28a plasmids were further transformed into *E. coli* BL21(DE3) cells for overexpression of *Ec*-3-MST wild type and C238A mutant proteins, respectively. R179L, R188L and R179L R188L mutants of *Ec*3-MST were cloned in a similar manner. Human MST protein, called *Hs*-MPST, was also sub-cloned from the pcDNA3.1+ vector obtained from Genscript (clone ID: OHu09558) into the pET28a vector. All clones were confirmed by sequencing using vector-specific T7 primers.

6.2.3 Growth conditions and media:

6.2.3.1 Primary culture set-up (LB and P1 cultures): LB and P1 cultures of all strains were set up in the same manner as previously described (Chapter 2, Materials and Methods, Section 2.2.4.1).

Sr. No.	Primer name/ purpose	Primer Sequence (5' → 3')
1.	*thiS::cam*^{R}_for	CCTGGCGTTGCGCCGCGCCAGTGGTTGCCCGGTATGCGG AGGAAGCAATGCATATGAATATCCTCCTTAG
2.	*thiS::cam*^{R}_rev	CAAACGTTTTGTCCGCAATACGTAACATTTCAACCCCCTG CAATAACCTGTGTGTAGGCTGGAGCTGCTTC
3.	*thiS::cam*^{R}_conf_for	TTAAGCGGTATAGAGACACC
4.	*cam*^{R}_conf_rev	AGTTGTCCATATTGGCCACG
5.	*EcsseA*_C238A_for	AAACCAATTATCGTCAGCGCGGGCTCTGGTGTAACGGCA
6.	*EcsseA*_R179L_for	GCCCGCCCGGCTGCACTGTTTAACGCAGAAGTT
7.	*EcsseA*_R188L_for	GAAGTTGATGAACCTCTGCCAGGTTTACGTCGC
8.	*EcsseA*_R179L R188L_for	GCCCGCCCGGCTGCACTGTTTAACGCAGAAGTTGATGAA CCTCTGCCAGGTTTACGTCGC
9.	*Hs*MPST_PCR1_for	ATGGCTTCGCCGCAGCTCTG
10.	*Hs*MPST_PCR1_rev	TCAGTGGGTCTTCCCCCGGC
11.	*Hs*MPST_PCR2_for	AGCCATATGGCTAGCATGGCTTCGCCGCAGCTCTG
12.	*Hs*MPST_PCR2_rev	AGCTCGAATTCGGATCCTCAGTGGGTCTTCCCCCGGC
13.	T7 Promoter_for	TAATACGACTCACTATAGGG
14.	T7 Terminator_rev	GCTAGTTATTGCTCAGCGG

Table 6.1 Sequences of primers used in Chapter 6. Key: for = forward primer, rev = reverse primer, conf = diagnostic PCR (confirmatory) primer, cam = chloramphenicol, *sseA* = 3-MST gene.

6.2.3.2 *Secondary culture set-up (P2 cultures):* P2 cultures and co-cultures were set up following the same washing protocol as previously mentioned (Chapter 2, Materials and Methods, Section 2.2.4.2). Following this, co-cultures of mutants in liquid M9-Glucose medium were set up using the same protocol as previously described (Chapter 3, Materials and Methods, Section 3.2.3.2). The medium was supplemented with or without either inosine (50 μM) or thiamin (20 μM) or both (total 4 conditions) unless mentioned otherwise.

6.2.3.3 *Primary and secondary culture set-up (LB and P1 cultures) for 3-MST project:* *E. coli* BL21(DE3) cells with either empty or *Ec*3-MST-pET28a vector were grown in LB aerobically at 37°C, 180 rpm, for 10 h, in 200 μL medium in a 96-well plate. These cells were washed with 1X M9 salts and used to start their growth curves in P1 cultures, at a starting OD_{600} of 0.05. The media used were [M9 + NH_4Cl + Glucose + Kanamycin (25 μM)] medium, with 100 μM of Isopropyl β-D-1-thiogalactopyranoside (IPTG), supplemented with or without varying concentrations of chloramphenicol (0 μM, 1 μM, 2 μM, 4 μM and 8 μM). Chloramphenicol solutions were prepared in distilled water, and not in ethanol. Stock solutions of the antibiotics were 20X concentrated than their final concentrations in the medium, and were prepared using two-fold serial dilutions.

6.2.4 Overexpression and purification of C238A EcsseA: Overnight primary cultures of *E. coli* BL21(DE3) cells containing the C238A *Ec*3-MST-pET28a plasmid were grown in LB with kanamycin (25 μg/ mL) at 37°C, 180 rpm. These cultures were transferred into large secondary cultures of LB with kanamycin (25 μg/ mL) medium at 1% v/v inoculum and were grown at 37°C, 180 rpm to an OD_{600} of 0.6. Protein overexpression was achieved with 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG), following which cultures were further incubated at 18°C for 18 h, 180 rpm. Cells were harvested from large cultures by centrifugation at 6500 rpm, 4°C and pellets were flash frozen in liquid nitrogen and stored at -80°C till further use. Cell pellets obtained were resuspended in the lysis buffer (50 mM Tris-HCl, pH 8.0) which contained 300 mM NaCl, 10 mM $MgCl_2$, 0.025% β-mercaptoethanol (BME), and 0.05 mM phenylmethylsulfonyl fluoride and were lysed by sonication. The cell lysate was loaded onto a 5 mL pre-packed Ni-NTA column (Bio-Rad) pre-incubated with 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl, 10 mM imidazole, and 0.025% BME. The column was then washed with 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl, 0.025% BME, and 50 mM imidazole, and the protein was eluted in 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl, 0.025%

BME, and 250 mM imidazole. Eluted protein fractions were separated on sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) to check for the presence of C238A *Ec*3-MST. The purified protein was buffer-exchanged in 50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 0.025% BME using Econo-Pac 10DG pre-packed desalting columns (Bio-Rad) and stored with 15% v/v glycerol at -80°C.

6.2.5 Isolation of the 'yellow compound' produced by the ΔpurK strain: The conditioned medium from the *E. coli* K-12 MG1655 *ΔpurK* strain grown in P2 cultures was used for isolation of the yellow compound. Briefly, the medium was treated with 4% HCOOH, and centrifuged at 14000 rpm, 4°C, for 1 h to precipitate the proteins present in it, if any. The supernatant was then passed through a 0.22 μM filter to remove any bacterial cells present, and was separated using a high-performance liquid chromatography (HPLC) system from Agilent (1260 Infinity II) on a C-18 reverse phase column (Phenomenex, Gemini, 5 μm, NX-C18, 110 Å, 250 × 4.6 mm), at 25°C. The solvents used were 100 mM potassium phosphate buffer, pH 6.6 in line A, MilliQ in line B, and methanol in line C. 50 μL – 500 μL of samples were injected for collections. The flow rate was maintained at 1 mL/ min. The method used was 0 min: 100% A; 2 min: 90% A, 10% B; 10 min: 15% A, 25% B, 60% C; 12 min: 15% A, 25% B, 60% C; 15 min: 100% A; 22 min: 100% A. The peak at 11.3 min was collected, vacuum-dried at RT and resuspended separately in water, dichloromethane, chloroform, ethyl acetate, hexane, *n*-butanol, and cyclohexane for obtaining UV-Vis spectra from 200 nm – 500 nm (Figure 6.8).

6.3 Research design, Results, and Discussion

6.3.1 Analyzing the role of ThiS

6.3.1.1 Generating the ΔthiS mutant of E. coli K-12 MG1655

E. coli str. K-12 substr. MG1655 *ΔthiS::cam*^{R} mutant was created using the protocol mentioned earlier (Table 6.1 – primers 1-2). Diagnostic PCRs were carried out to verify gene deletion and simultaneous insertion of the chloramphenicol cassette using gene specific primers. The WT strain used as a negative control for colony PCR showed no product as it lacks the chloramphenicol cassette for the reverse primer to bind, but the deletion mutant showed product of expected length at around 900 bp (Figure 6.5, Table 6.1 – primers 3-4).

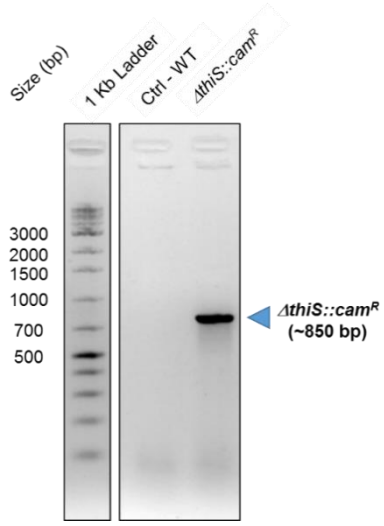

Figure 6.5 Diagnostic PCR for verifying the *ΔthiS* mutant. Control reaction with the WT strain does not show amplification, whereas that with the *ΔthiS* mutant shows deletion-specific product.

6.3.1.2 Growth phenotype of the ΔthiS mutant

The *ΔthiS* mutant of *E. coli* was analyzed for its growth phenotype in the M9-glucose minimal medium in the second passage P2. We observed that the *ΔthiS* mutant fails to grow in the P2 passage in the absence of thiamin, but it can grow when supplemented with either THZ or thiamin (Figure 6.6A). This confirmed that this mutant is indeed defective for THZ biosynthesis.

6.3.1.3 Co-cultures of thi⁻ mutants with the ΔthiS mutant

Next, we analyzed the growth of *CS, ES* and *GS* co-cultures of *E. coli* in M9-glucose medium in the P2 passage in the presence and absence of thiamin. We hypothesized that the *CS* co-culture might survive if the *ΔthiS* strain supplements the *ΔthiC* strain with HMP, and the *ΔthiC* strain which can synthesize thiamin using the supplemented HMP provides thiamin back to the *ΔthiS* strain, similar to our observations for the *CG* co-culture in Chapter 3 (Figures 3.1C and 3.2B). We further hypothesized that the *ES* co-culture might not survive, similar to the results obtained for the *EG* co-culture in Chapter 3, which show that THZ does not get exchanged in co-

cultures of *E. coli thi*$^-$ mutants. As both *ΔthiG* and *ΔthiS* strains are defective for THZ biosynthesis, we also hypothesized that the *GS* co-culture would not survive. We indeed observed that the CS co-culture survives in the absence of external thiamin supplementation in the P2 passage of M9-glucose medium (Figure 6.6B). We also observed that *ES* and *GS* co-cultures failed to grow in the absence of exogenous thiamin in the P2 passage of M9-glucose medium (Figures 6.6C and 6.6D). This further confirms that THZ is indeed not exchanged in co-cultures of *E. coli thi*$^-$ mutants.

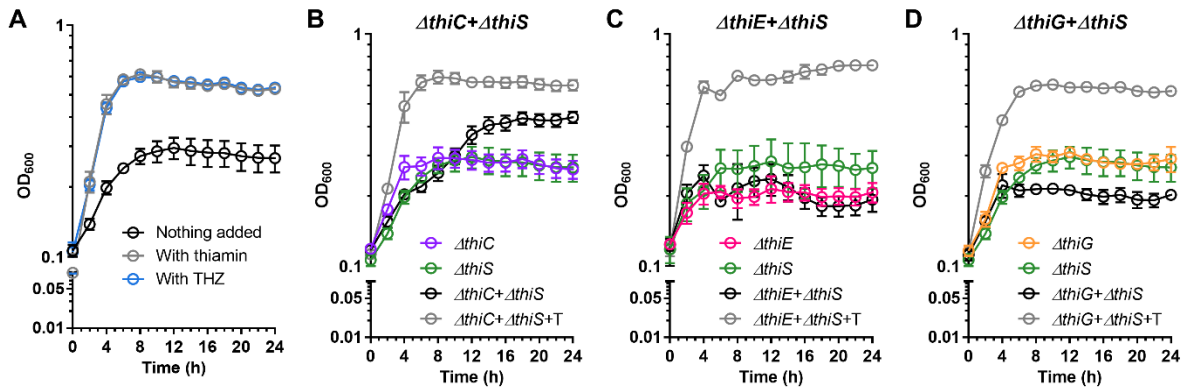

Figure 6.6 Growth phenotypes of cultures of thiamin biosynthesis mutants of *E. coli*. (A) Growth phenotype of the *ΔthiS* strain with thiamin (20 µM) and thiazole (20 µM), (B) *CS*, (C) *ES*, and (D) *GS* co-cultures in the P2 passage. Means ± standard errors of the means from three independent experiments are plotted. + T = with thiamin.

6.3.2 Analyzing the role of 3-mercaptopyruvate sulfurtransferase (3-MST) in E. coli

We analyzed the role of artificial substrates of 3-MST synthesized by Chakrapani lab, in generating H_2S and persulfides. Cloning and purification of C238A *Ec*3-MST and sub-cloning and purification of *Hs*MPST was carried out in our lab. We showed that these substrates can be used by 3-MST to increase intracellular persulfides, which can protect against neuroinflammation[44]. We next analyzed the effects of *Ec*3-MST on the physiology of *E. coli*. We hypothesized that H_2S produced *Ec*3-MST protein might alleviate chloramphenicol stress in *E. coli*. To analyze this, we

cultured *E. coli* BL21(DE3) cells with either the empty pET28a plasmid or the *Ec*3-MST plasmid in M9-glucose minimal medium in the P1 passage with IPTG, in the presence of chloramphenicol. Under these conditions, when the strain carrying *Ec*3-MST was challenged with chloramphenicol, it showed an advantage in growth compared to the one carrying an empty plasmid (Figure 6.7). This might mean that overexpressed *Ec*3-MST generated H_2S *in vivo*, which helped in alleviating antibiotic stress. We also created C238A, R179L, R188L, and R179L R188L mutants of *Ec*-3-MST enzyme and *E. coli* K-12 MG1655 *ΔsseA* and *ΔycjW* strains, and are currently investigating their activity in the presence of artificial substrates and inhibitors made by Chakrapani lab.

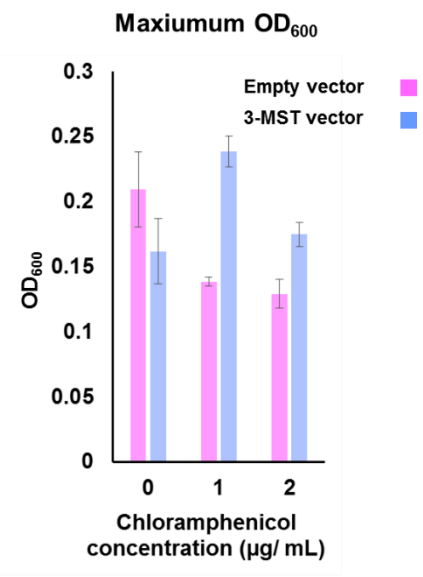

Figure 6.7 Antibiotic-stress and *Ec*3-MST. *E. coli* BL21(DE3) cells with either empty or *Ec*3-MST pET28a vector grown in the P1 passage of M9-glucose minimal medium, in the presence of varying chloramphenicol concentrations. Values represent maximum OD_{600} attained during 10 h of growth. Means ± standard errors of means from three independent experiments are plotted.

6.3.3 Analyzing the 'yellow compound' produced by the ΔpurK strain

We also analyzed the yellow compound produced by the *ΔpurK* mutant of *E. coli*, which seems to be linked to the accumulation of AIR. Similar to the P1 passage, we observed that only

the *ΔpurK* strain produces a yellow-coloured compound in the P2 passage, whereas the rest of the thiamin and purine mutants do not (Figure 6.8A). Also, this yellow compound seems to be produced only under aerobic conditions, and not under anaerobic conditions (Figure 6.8B). Thus, oxidation of accumulated AIR might be the cause of this colour as observed earlier for yeast[36,37,40].

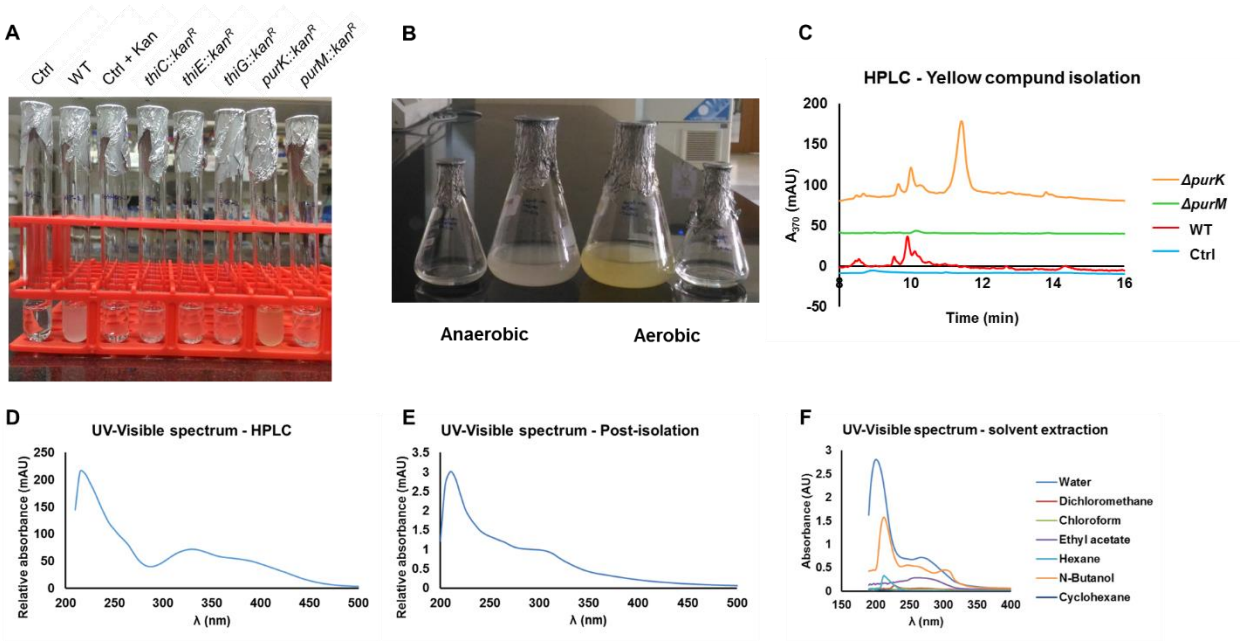

Figure 6.8 Analyzing the yellow compound produced by the *ΔpurK* mutant. (A) *E. coli* thiamin and purine mutants after 24 h of growth in the P2 passage of M9-glucose medium.

We further separated the spent medium of the *ΔpurK* mutant on HPLC, and using the spent media from WT and *ΔpurM* strains, identified this compound as a distinct peak present only in the spent medium from *ΔpurK* strain, at ~11.3 min (Figure 6.8C). The UV-visible spectrum of the yellow compound shows peaks at about 215 nm, 260 nm, 330 nm and 375 nm in the HPLC for the peak at 11.3 min (Figure 6.8C-E). As this compound is yellow in colour, and the absorbance maxima are shifted to the left from the usual absorption wavelength for the yellow colour (around 400 nm), these results show that it might be resonance stabilized and have partially aromatic nature. We also performed solubility assay for the isolated compound using UV-visible spectrum-

based analysis (Figure 6.8F). We found the yellow compound to be soluble in polar solvents such as water and *n*-butanol, with partial solubility in ethyl acetate, but it was almost insoluble in hydrophohic solvents such as dichloromethane, chloroform, hexane and cyclohexane. This shows that it also has some polar groups in addition to partial conjugation/ aromaticity. As AIR is aromatic, the amino acids reported to attach to it upon its accumulation are both hydrophobic and hydrophilic, and the spectra reported for those compounds show multiple peaks like those obtained by us, this yellow compound might indeed be the AIR oxidation product as observed earlier[36,37,40].

6.4 Summary

We observed that the *CS* co-culture of *E. coli* survives in the absence of thiamin in M9-glucose minimal medium, whereas *ES* and *GS* co-cultures do not. These results reiterate our findings from Chapter 3 and 4, which show that HMP and thiamin get exchanged among *E. coli* mutants, whereas THZ does not. But the growth observed for the *CS* co-culture appears to be lesser than that for the *CG* co-culture (Figure 3.2B and 6.6B). This might point to differences in the physiology of thiamin mutants, even when they share the same branch of the pathway.

We also analyzed the growth of *E. coli* BL21(D3) cells with overexpression of *Ec*3-MST protein, which seemed to alleviate chloramphenicol stress to some extent. This points to the protective role of H_2S produced by 3-MST from *E. coli* against antibiotic stress. The overall low growth observed here for these cells might be due to added stress of IPTG in the M9-glucose minimal medium. We are studying the activity of *Hs*MPST and other mutants of *Ec*3-MST with artificial substrates and inhibitors to gain more insights into the physiological role of this protein.

During our experiments, we observed a yellow compound produced by the *ΔpurK* mutant of *E. coli* K-12 MG1655. Yeast mutant strains that are deficient for homologues of this gene have been shown to produce such coloured pigments as a result of oxidation of AIR[36,37,40]. Our analysis of this molecule also points to it being the yellow-coloured AIR oxidation product. This compound is related to other moieties in cells such as iron, amyloids, and has been used as a genetic marker for mutation-based assays[45–47]. Differences observed in the colour of this compound in different organisms might point to differences in physiology and flux through the same metabolic pathway.

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Chapter 7: Perspectives and future directions

7.1 Summary

In our work, we analyzed the exchange of nutrients among thiamin biosynthesis mutants of *E. coli* K-12 MG1655. We observed that only *CE* and *CG* co-cultures of *thi*$^-$ mutants can survive in M9-glucose minimal medium without exogenous thiamin supplementation, whereas the *EG* co-culture does not grow. Based on this data, we inferred that thiamin and its biosynthesis intermediate HMP are readily exchanged in synthetic co-cultures of *E. coli thi*$^-$ mutants, whereas THZ cannot be exchanged. This data is in line with several other reports in literature which either predict or experimentally prove – (i) prevalent exchange of HMP and thiamin, (ii) presence of free HMP and higher number of HMP auxotrophs in natural ecosystems, and (iii) more transporters for HMP and thiamin as compared to THZ. Additionally, we observed that the three-membered community of *E. coli thi*$^-$ mutants also survived in the absence of exogenous thiamin supplementation in M9-glucose minimal medium.

We further analyzed the exchange of intermediates between purine and thiamin biosynthesis pathway mutants of *E. coli* K-12 MG1655 as these two pathways share a common intermediate, AIR. Here, we observed that only the *CK* co-culture can survive in the absence of exogenous inosine and thiamin supplementation in M9-glucose minimal medium, whereas *EK*, *GK*, *CM*, *EM*, and *GM* co-cultures do not grow. We also observed that in M9-glucose medium supplemented with inosine, *EM* and *GM* co-cultures can survive, whereas the *CM* co-culture does not grow. Taken together, these results further implicate the exchange of HMP, thiamin, and inosine or some other purine intermediate in synthetic co-cultures of *E. coli*.

We also calculated the ratio of mutants in the co-cultures that survived, and observed that in two- or three-membered co-cultures of *thi*$^-$ mutants, the *ΔthiC* strain increases in number and attains about 80% of the final population in the absence of exogenous thiamin supply. It thus maintains a population of either the *ΔthiE* or the *ΔthiG* strain or both these strains, which is about 1/4th of its size, by supplementing them with thiamin in return for a small, but continuous supply of HMP. We also observed that the ratio of thiamin mutants in the pairwise co-cultures does not

deviate much from the starting ratio when thiamin is supplemented. This indicates when thiamin is available, these strains do not interact with each other, as they can assimilate thiamin and survive on their own. In case of the *CK* co-culture, we observed that the percentage of the *ΔthiC* strain increases to about 40%-50% by 10 h, and decreases thereafter. This might suggest a competition for nutrients among the strains as they belong to two different pathways and hence require two different types of nutrients for their survival.

We also visualized the spatial organization of thiamin mutants in two- and three-membered co-cultures. We observed that in the absence of exogenous supply of thiamin, these mutants co-localize with each other, whereas in its absence, they do not co-localize and rather grow separately, forming invaginations. This indicates that they interact when thiamin is absent and hence co-localize to facilitate the interactions. We also visually observed a reversal of ratios of the two mutants in pairwise co-cultures when thiamin is absent, as compared to the co-cultures supplemented with thiamin.

This study provides a method to analyze pathways that are interconnected with shared metabolites such as 5'-phosphoribosyl-5-aminoimidazole (AIR), 1-deoxyxylulose-5-phosphate (DXP), chorismate and α-ketoglutarate/ glutamate[1]. Studies such as this help analyze the 'effective affected metabolism' in a consortium of interacting microbes which shapes the community dynamics, beyond what is studied in a single organism.

7.2 Future perspectives

Based on the results obtained by us, and other observations in literature, certain questions arise that still require a detailed analysis. We discuss some of those questions below and propose certain experiments that can be performed.

7.2.1 Presence of transporters

We observed that *E. coli* can uptake THZ at concentration as low as 1 nM, whereas HMP uptake appears to happen at 100 nM. But *E. coli* does not have any reported transporters of HMP and THZ, and contains only one transporter for thiamin. Hence, it is yet unclear if HMP and THZ transport in *E. coli* occurs via a passive diffusion or via active transporters. If the transport occurs via passive diffusion, it would also be interesting to analyze if the differences in concentrations at

which HMP and THZ are picked up are reflective of their chemical nature such as solubility or functional groups. As HMP is more hydrophobic than THZ, one would predict its uptake to be preferential than THZ due to the ease of passing through the lipophilic membranes of bacteria.

7.2.2 Exchange of THZ and flux in HMP and THZ branches

From our experiments, we observed that THZ exchange in *E. coli* co-cultures is restricted. An earlier report in *Salmonella typhimurium*, another gram-negative gammaproteobacterium like *E. coli*, shows that its thiamin-starved HMP auxotroph shows thiamin production as soon as HMP is supplemented, but does not contain THZ in its absence[2]. This shows that this auxotroph has the ability to make THZ quickly when supplemented with HMP, but does not make it when not needed. Such studies show that only specific metabolites might have properties that make their exchange feasible, and pose the need to calculate the metabolite flux in the two branches of the pathway. This study, when correlated with the concentrations at which HMP and THZ are picked up by *E. coli*, might explain the need of HMP supplementation at a concentration higher than that of THZ, provided that HMP is produced more efficiently than THZ.

7.2.3 Effect of mutations in other THZ biosynthesis pathway genes

We observed that *CS* and *CG* co-cultures of *E. coli* survive in the absence of thiamin. Both ThiG and ThiS are involved in THZ biosynthesis, but they play different roles; ThiG assembles the precursors of THZ, whereas ThiS helps in sulfur transfer. Apart from these, THZ biosynthesis involves other proteins, and it would be interesting to analyze their role in the exchange of intermediates in thiamin biosynthesis pathway, by co-culturing their mutants with the *ΔthiC* strain.

7.2.4 Specific interactions in the three-membered community of thiamin mutants

We observed that a three-membered community of *ΔthiC*, *ΔthiE*, and *ΔthiG* strains survives in the absence of thiamin, but the nature of interactions in this community yet remains elusive. It would be interesting to analyze the effect of perturbations such as passaging it further in M9-glucose medium or supplementation with precursors that specifically affect one of the two branches of thiamin biosynthesis pathway.

7.2.5 Identifying the purine intermediate exchanged between purine and thiamin mutants

We observed that the *CK* co-culture of *E. coli* survives in the absence of external supply of inosine and thiamin. But the purine intermediate exchanged between them yet remains unidentified. For this, the co-cultures of the *ΔthiC* strain with mutants of other genes on the purine biosynthesis pathway that appear after the *purK* gene could be analyzed.

7.2.6 Regulation of exchange between purine and thiamin mutants

The PurR regulon of *E. coli* regulates expression of genes by forming a complex of hypoxanthine with the PurR protein when purines are available. This regulon has been shown to upregulate the expression of both *thiC* and *thiE* genes in *E. coli*[3]. This would mean that upon purine supplementation by thiamin mutants in a co-culture, the expression of both these genes should be upregulated in the *ΔpurK* strain. In that case, the *ΔpurK* strain should be able to produce equivalent amounts of HMP and thiamin, and should supplement *ΔthiE* and *ΔthiG* strains as well. But this does not occur, and it would be interesting to analyze the extent to which the limitation of flux in the THZ biosynthesis branch of the pathway affects thiamin biosynthesis in the *ΔpurK* mutant. Similarly, exploring the effects of other pathways such as that of histidine biosynthesis which interact with the purine biosynthesis pathway would be interesting[1].

7.2.7 Identifying the nature of interaction during the co-localization of mutants

In our experiments, we observed that thiamin mutants of *E. coli* co-localized in the absence of external thiamin supplementation when grown as a co-spot. At any given point in the co-spot, the mutant strains might co-exist and as the local concentration of one of the nutrients increases, growth of the strain which requires that nutrient gets facilitated at that spot. Thus, such a co-localization seems to be a passive phenomenon. In future, to verify whether thiamin biosynthesis mutants actively co-localize, chemotaxis mutants of *E. coli* can be used.

7.3 References

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