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Heat shock and chemical-induced stress response of *Pseudomonas putida* strains

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Heat shock and chemical-induced stress response of *Pseudomonas putida* strains

Abstract:

The C1 chemicals, such as formate and methanol, are potential feedstocks for sustainable bioproduction. This thesis investigates whether microorganisms that have been genetically modified to assimilate these substances through the reductive glycine pathway face stress. The growth experiments in C1 substrates under heat shock and chemical stress conditions by using stress response reporter systems, pAG032 and pBSibp_Amp, were performed. pBSibp_Amp is a better choice for measuring the heat shock response because it gives much higher ratios than pAG032, and fluorescent bacterial proteins, naturally produced by *P. putida* and the red fluorescent protein's fluorescence spectra from plasmid do not overlap. Genetically modified strains can tolerate high concentrations of methanol and formate but do not yield biomass in these growth conditions.

Keywords: *Pseudomonas putida*, C1 compounds, heat shock response, chemical-induced stress response, stress response reporter systems.

CERCS: B230 Biomedical Sciences Microbiology, bacteriology, virology, mycology.

Kuumashoki ja keemiliste ühendite poolt indutseeritud stressi uurimine *Pseudomonas putida* tüvedes

Lühikokkuvõte: C1-ühendite, metanooli ja formiaadi, kasutamine tööstuslikus biotehnoloogias ja bioenergia tootmises on jätkusuutlikus ringmajanduses oluline uurimissuund. Väga vähesed mikroobid suudavad kasutada neid ühendeid süsiniku- ja energiaallikana. Käesolevas töös uuritakse kuumašoki ja kemikaalide põhjustatud stressi mikroorganismidel, mida on geneetiliselt muundatud C1 ühendite assimileerimiseks redutseeriva glütsiini raja kaudu. Selleks kasutati reportersüsteeme pAG032 ja pBSibp_Amp. Kuumašoki stressivastuse mõõtmiseks sobib paremini pBSibp_Amp, sest sellega saadi palju kõrgemad väärtused kui pAG032 reporteriga ning *P. putida* toodetavate fluorestseeruvate valkude ja reporteri punase fluorestseeruva valgu (RFP) spektrid ei kattu. Geneetiliselt muundatud tüved taluvad kõrgeid metanooli ja formiaadi kontsentratsioone, kuid biomassi saagis ei suurene.

Märksõnad: *Pseudomonas putida*, C1 ühendid, kuumašoki vastus, kemikaali-indutseeritud stressivastus, stressivastuse reprtersüsteemid.

CERCS: B230 Biomeditsiin Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia.

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ABBREVIATIONS

Amp - ampicillin
Bp - benzylpenicillin
Bp^R - benzylpenicillin resistant
CFP - cyan fluorescent protein
HS - heat shock
Km - kanamycin
Km^R - kanamycin resistant
LB - Luria Bertani medium
Minimal - minimal medium, contains glucose, cas amino acids, serine, and tryptophan
mQ - “ultrapure” water
Pseudomonas putida KT2440 - wild type or KT2440
Pseudomonas putida KT2440_C1-S-Aux - CSA
Pseudomonas putida KT2440_C1-S-Aux_pMnecator - CSA_pMnec
Pseudomonas putida KT2440_C1-S-Aux_pMnecator+pAG032 - CSA_pMnec+pAG032
Pseudomonas putida KT2440_C1-S-Aux_pMnecator+pBSibp_Amp - CSA_pMnec+pBSibp
Pseudomonas putida KT2440_C1-S-Aux+pAG032 - CSA+pAG032
Pseudomonas putida KT2440_C1-S-Aux+pBSibp_Amp - CSA+pBSibp
Pseudomonas putida KT2440+pAG032 - KT2440+pAG032
Pseudomonas putida KT2440+pBS(T)ibp_Amp - KT2440+pBSibp
Pseudomonas putida KT2440+pBSibp_Amp - no fluorescence, negative control or NC
rGlyP - Reductive Glycine pathway
RFP - red fluorescent protein
wt - Wild-type
YFP - yellow fluorescent protein

INTRODUCTION

Since the beginning of the industrial revolution, the world has faced the issue of large amounts of outputting waste, causing soil, air and water pollution with gases and chemicals. With the continuation of urbanisation and other industrial activities, the environment and humanity are at risk. Traditional methods of remediation include the use of harsh chemicals and burning, but these are high-cost, disruptive, and still, slower but pollution-causing.

Different microbial feedstocks such as formate and methanol can be employed as C1 substrates for the biobased synthesis of chemicals and fuels. Regretfully, *Pseudomonas putida* and other aerobic cell factory organisms are unable to utilize C1 substrates naturally. The most effective approach is to use genetically modified bacteria with introduced pathways, natural or synthetic, to convert feedstock to valuable products. This is a new era of cell factories and the recycling of bioresources allowing ecologically friendly and continuous utilisation of otherwise toxic substances.

This work is focused on the measurement of heat shock and chemical-induced stress responses in the model organism *Pseudomonas putida* KT2440 and in genetically modified strains of *P. putida* KT2440 possessing reductive glycine pathway for the assimilation of toxic and hazardous chemicals: methanol and formate. It is important from the point of view of future industrial applications to know the resistance and stress response of bacteria to sudden temperature or substrate concentration rise. To fulfill this goal two plasmid-based reporter systems, pAG032 and pBSibp_Amp were used.

This thesis is a part of the EU-funded Horizon program project ‘BIOS: The bio-intelligent DBTL cycle, a key enabler catalysing the industrial transformation towards sustainable biomanufacturing’, which focuses on the introduction of circular bioeconomy, by converting sustainable substrates through efficient bioprocessing. To advance the efficiency of DBTL ('design-build-test-learn') cycle, several improvements, such as hybrid learning or the creation of digital twins, are introduced. This method is tested on the engineering of the *Pseudomonas putida* strains, which will be capable of producing terpenes, polyolefins, and methylacrylate, all of which have significant potential to reduce greenhouse gas emissions.

1. LITERATURE OVERVIEW

1.1 Bacterial stress tolerance and response

One of the main challenges in contemporary microbiology in relation to biotechnological applications is the need to understand the processes of stress tolerance of microorganisms. By studying these processes, the key components of stress response pathways and networks can be discovered and this knowledge can be used to overcome the natural susceptibility of bacteria to harsh chemicals, high temperature or pH changes (Matin, 2014), creating bacteria, which might not just be able to become more stress-sustainable, but also improve the overall performance under stress-induced conditions (Bruinsma et al., 2023). This might be advantageous in fermentation-related processes in cell factories (Stanbury et al., 2017) in cases of breakages and accidents, as well as in the bioremediation field (Azubuike et al., 2016), which is still evolving and slowly but steadily leads to more "green" and safe waste utilization options by reducing harsh chemical, heavy metal, and greenhouse gases pollution (Azubuike et al., 2016).

Gram-negative bacteria's stress response is primarily regulated by specific sigma factors, including σ^{54}, σ^{70}, and group 2 σ^{S}, which orchestrate transcriptional responses (Lange and Hengge-Aronis, 1991; Fang et al., 1992). These responses are crucial for developing stress-induced resistance to various environmental stressors, such as heat, cold, pH extremes, oxidation, hyperosmosis, and exposure to antibiotics and antimicrobials (Matin, 2001).

Stress responses encompass protein repair mechanisms involving chaperones like DnaK, DnaJ, GrpE, GroEL, and GroES (Hendrick and Hartl, 1993), along with DNA repair enzymes such as Endonuclease III and IV, Dps (PexB), AidB, and the involvement of DnaK in DNA repair pathways (Hoeijmakers, 1991).

Sigma factors recognize consensus promoter sequences, with σ^{54} controlling genes involved in diverse functions such as starvation, flagellar synthesis, and growth on non-preferred substrates (Matin, 2014). σ^{S}, crucial in inducing the general stress response (GSR), governs the expression of core stress genes under diverse stress conditions. Ancillary regulatory molecules, such as cyclic AMP (cAMP) and guanosine tetraphosphate (ppGpp), modulate stress responses by facilitating transcription via σ^{70} and enhancing σ^{S} binding to the RNA polymerase core enzyme (Matin, 2014).

Transcriptional control by alternative sigma factors predominates in regulating stress responses, with genes or operons within specific response regulons possessing promoters recognized by distinct sigma factors whose activities correlate with environmental conditions

triggering the response. Repressor binding to DNA control elements is another mechanism, with HrcA repressor binding to the conserved inverted repeat control element "CIRCE" upstream of operons encoding heat shock proteins in both Gram-positive and certain Gram-negative bacteria (Dworkin et al., 2006).

Transcriptional control through proteolysis plays a significant role in stress response regulation, exemplified by SOS response to genotoxic effects mediated by autoregulated proteases. Small RNAs have been recognized as a vital regulatory mechanism, modulating the cellular concentration of RpoS (σ^{38}), the alternative σ factor involved in the starvation response and regulating the response to oxidative stress (Dworkin et al., 2006).

1.1.1 Heat shock response

The heat shock response is the first ever identified global regulation network, being the most fundamental one. This phenomenon is universal, present in every life organism ever studied, enhancing thermotolerance through homeostatic and protective cellular processes (Craig, 1985). A sudden rise in ambient temperature that causes the activation of a broad class of proteins known as heat shock proteins (HSPs), is what defines the heat shock. HSPs protect cells against damage which is caused by high temperatures and they also produce tolerance against high salt and heavy metals concentrations. (Dworkin et al., 2006).

Table 1. Factors affecting the regulation of major stress genes (adapted from Dworkin et al., 2006).

Gene	Function of gene product	Bacteria	Regulon	Transcription during stress	Control element	Stability of gene product
DnaK	Chaperone	Gram positive	Heat shock	σA (σ70)	CIRCE	
		Alphaproteobacteria	Heat shock	σ32		
		Gammaproteobacteria	Heat shock	σ32		
GroEL	Chaperone	Gram positive	Heat shock	σ70	CIRCE	
		Alphaproteobacteria	Heat shock	σ32	CIRCE	
		Gammaproteobacteria	Heat shock	σ32		
rpoH	Activator—σ32	Alphaproteobacteria	Heat shock	σ32		Stable
		Gammaproteobacteria	Heat shock	σ32, σE		Unstable
lon, clpP	Proteases	Gram negative	Heat shock	σ32		
		Gram positive	General stress	σB		

Abbreviation: CIRCE, a conserved inverted repeat control element or "controlling inverted repeat of chaperone expression."

HSPs are molecular chaperones and ATP-dependent proteases that are very important in protein folding and degradation processes, either under normal physiological conditions or during stress responses. (Sherman and Goldberg, 1992; Sherman and Goldberg, 1996; Kandror et al., 1994). GroEL and DnaK (Table 1) are highly conserved across evolutionary scales, meaning that their roles in cellular homeostasis and biological systems are extremely essential (Boorstein et al., 1994; Gupta, 1995). The regulation of proteins involved in protein

folding, refolding, quality control, and degradation is tightly orchestrated in bacteria. This regulation is ruled by heat shock sigma factors, with σ^{32} and σ^{E} playing the most vital roles.

The first regulatory system involves alternative sigma factors, which act as transcriptional activators, recognizing specific heat shock promoters upstream of heat shock genes (Ron, 2013). In Gram-negative bacteria, like *P. putida*, σ^{32} and σ^{E} are key players in this process, initiating the transcription of heat shock genes and ensuring the timely expression of proteins crucial for cellular stress response (Morita et al., 2000).

The second regulatory system employs transcriptional repressors, with HrcA being the most conserved and meaningful among them. HrcA binds to a conserved regulatory element called CIRCE located upstream of heat shock operons (Ron, 2013).

Sigma-32 (σ^{32}) is a pivotal regulator in Gram-negative bacteria's heat shock response, for instance, orchestrating the expression of at least 34 heat shock genes in *Escherichia coli* (Richmond et al., 1999; Morita et al., 2000). Under severe heat shock, σ^{E} activates the transcription of *rpoH*, encoding σ^{32}, and autoregulates its own expression via an σ^{E} promoter. This response is intricately regulated by various factors, including RseA, RseB, DegS, and YaeL. (Alba and Gross, 2004).

1.1.2 ppGpp-mediated stress response

The nucleotide alarmone (p)ppGpp is a crucial mediator of stress responses in bacteria, particularly during challenges to outer membrane biogenesis and ADP synthesis. Its regulatory effects involve binding to RNA polymerase, leading to a comprehensive reprogramming of gene expression in response to stressors like amino acid starvation (Roghanian et al., 2019).

This rewiring, involving approximately 500 genes, can induce slow growth or dormancy, indicating an adaptive response to adverse conditions (Durfee et al., 2008). (p)ppGpp's role extends beyond stress, influencing bacterial physiology and pathogenesis, facilitating host invasion and conferring antibiotic tolerance (Dalebroux et al., 2010). It also responds to environmental stresses like carbon source starvation, fatty acid depletion, phosphate limitation, and heat shock (Hauryliuk et al., 2015).

The production of (p)ppGpp is tightly regulated during transcriptional repression of genes like *adk*, *lptA*, and *lpxA* (Roghanian et al., 2019), enabling bacteria to adapt and survive in challenging environments (Battesti et al., 2011; Goormaghtigh et al., 2018).

(p)ppGpp is essential for stress response, especially when interacting with lipopolysaccharide and ADP synthesis. The Tas1 enzyme was identified in *Pseudomonas aeruginosa*, which

codes for an antibacterial toxin. Tas1 produces (p)ppApp instead of (p)ppGpp, adding a new dimension to understanding alarmone-mediated stress responses (Ahmad et al., 2019). The binding sites of (p)ppGpp on RNA polymerase have been elucidated, leading to selective upregulation of stress response genes and downregulation of growth-related genes, enabling bacteria to adapt and survive in adverse conditions (Ross et al., 2016).

Basal levels of (p)ppGpp are essential for maintaining metabolic balance during adaptation and exponential growth phases, preventing excessive energy consumption and toxic accumulation of metabolic byproducts (Kundra et al., 2020). However, entry into the stationary growth phase or exposure to adverse conditions triggers rapid accumulation of (p)ppGpp, leading to the stringent response (SR), which orchestrates a comprehensive remodelling of cell physiology (Kundra et al., 2020).

RelA is the major synthetase in *Pseudomonas putida* responsible for the accumulation of (p)ppGpp (Schäfer et al., 2020). When treated with extreme heat or antibiotics, *P. putida* triggers the accumulation of (p)ppGpp, initiating cellular responses and adaptive mechanisms to cope with heat- and antibiotic-induced stress (Schäfer et al., 2020).

Spx is a transcription factor that plays a crucial role in the heat shock response of bacterial cells (Hecker et al., 1996; Elsholz et al., 2017). It interacts with RNA polymerase, activating redox chaperones and inhibiting transcription of genes related to competence and motility (Rochat et al., 2012; Molière et al., 2016; Schäfer et al., 2018). By binding to the C-terminal domain of the RNA polymerase alpha-subunit, Spx modulates gene expression and enhances the recognition of promoters, leading to stress response gene activation (Nakano et al., 2001, 2003). Spx also operates in a negative feedback loop, positively regulating *clpX* and *yjbH* gene expression (Larsson et al., 2007).

1.1.3 Stress response reporter system - pAG032

During the processes running in industry, a situation, where it is very crucial to monitor bacterial state, may occur. The accidents related to the availability of feedstock or harmful chemical leakage, or even the arbitrary temperature rise (Gawin et al., 2019) should be avoided at all costs, as they may harm not only the industrial processes but also the environment in some cases. That is why there is a need for an immediate and proper solution for monitoring the bacterial cell's state.

A plasmid-based reporter system, pAG032 (Fig. 1), was developed by Gawin et al., 2019 using the broad-host range replicon RK2 (Blatny et al., 1997), which includes promoters controlling the synthesis of three spectrally separable fluorescent proteins: mCherry (RFP), mVenus (YFP), and mCerulean (CFP) (Cox et al., 2010).

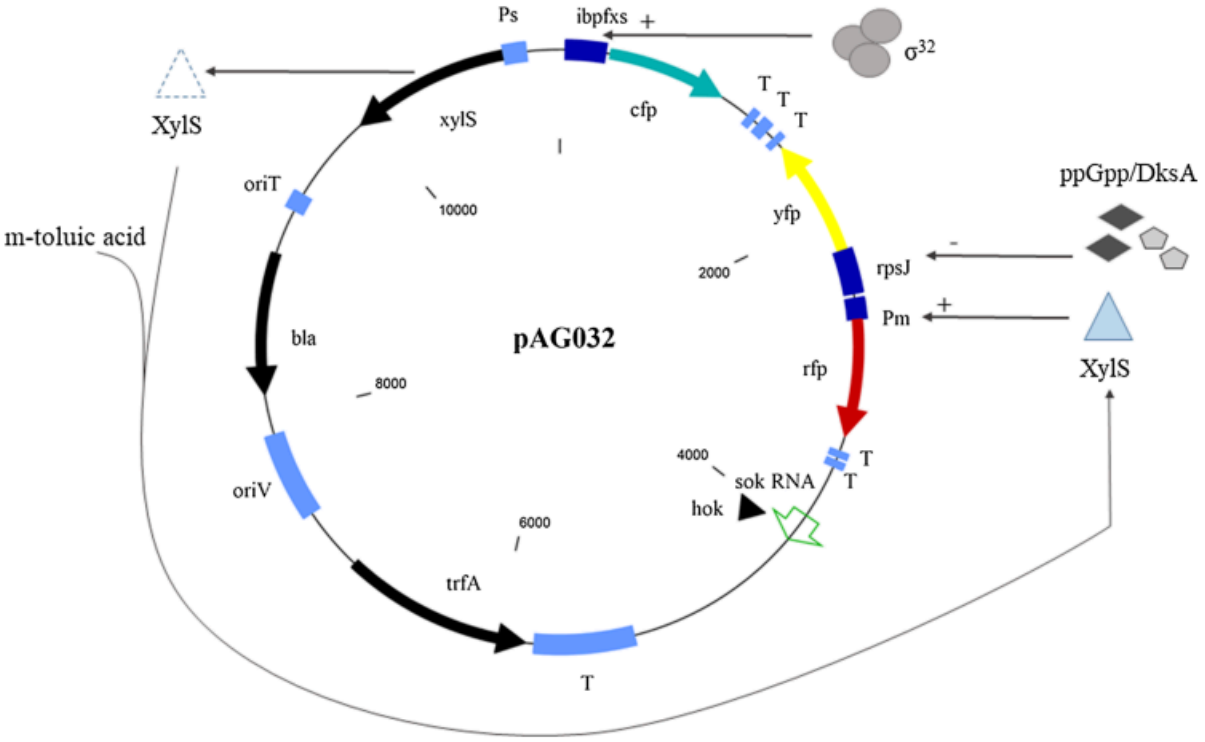

Figure 1. The three promoter/reporter pairs' regulatory mechanisms are depicted in the genetic map of the 11,238 nt-large pAG032 reporter plasmid. Benzoic acid derivatives, like m-toluic acid, can induce the XylS/*Pm* regulator/promoter, whereas intracellular concentrations of ppGpp and σ^{32} can activate the P*rpsJ* and P*ibpfxs* promoters, respectively. *oriT* stands for origin of conjugative transfer; *bla* is the ampicillin resistance gene; *trfA*, the gene encoding the crucial initiator protein TrfA; T, transcription terminators; *hok/sok* RNA, *hok-sok* suicide elements; Ps, σ^{70}-dependent constitutive promoter; *oriV*, origin of vegetative replication; The positive transcriptional regulator XylS is encoded by the gene *xylS*. (adapted from Gawin et al., 2019).

The system uses the inducible XylS/Pm regulator/promoter system from *Pseudomonas putida* TOL plasmid pWW0 to regulate RFP (587/610 (Seefeldt et al., 2008)) production (Gawin et al., 2019). The P*rpsJ* promoter, ppGpp-regulated ribosomal protein promoter, which coordinates the transcription of the S10 operon encoding 30S ribosomal subunit, of the *rpsJ* ribosomal protein gene was selected to control YFP (515/528 (Rekas et al., 2002)) expression and can monitor stringent stress response. The P*ibpfxs* tandem promoter, of *ibpAB*, which encodes two heat shock proteins involved in response to heat and superoxide stresses (Kitagawa et al., 2000), and *fxsA*, which encodes for inner membrane protein whose expression is associated with accumulation of misfolded proteins (Lesley et al., 2002), was used to monitor CFP (433/475 (Goedhart et al., 2010)) heat shock-like responses (Kraft et al., 2007). The study's reporter genes' distinct transcripts most likely do not express themselves independently. For instance, the fact that the σ^{32}-mediated heat shock response may cause the synthesis of ppGpp (Farr and Kogoma, 1991; VanBogelen et al., 1987), which inhibits P*rpsJ*, or the influence of the inducer on the overexpression of stress-responsive promoters are indications of a dependability. The plasmid's copy number can be adjusted to different numbers, including above 100 copies per genome, by replacing *trfA* with available mutant versions (Figurski et al., 1979). pAG032 also contains *oriT* for conjugative transfer and *hok-sok* suicide elements, ensuring segregational stability even under high-cell density cultivations (Sletta et al., 2004).

1.2 Bacterial degradation of methanol

Replacing sugars as substrates for bioproduction with C1 compounds, for example, methanol, will reduce greenhouse gas release and valorize the production of value-added chemicals (rhamnolipids, polyhydroxyalkanoates, cis,cis-muconate, etc.) (Turlin et al., 2023). Methanol is a very toxic compound to bacteria (Bennett et al., 2021), as it disrupts the cell membrane structure and functions by intercalation with fatty acids (Gustafson and Tagesson, 1985; Sonmez et al., 2013), and causes protein and nucleic acid damage by the transformation process of methanol to formaldehyde, which is extremely toxic (Teng et al., 2001). However, methanol can be used as a feedstock for methylotrophic bacteria (Turlin et al., 2023) and, consequently, for the production of energy, since, for instance, *E. coli* is able to handle up to 10% (2.5 M) of methanol (Ganske and Bornscheuer, 2006) and *P. putida* strain EM42 - up to 1.35 M (Turlin et al., 2023). The use of methylotrophs and genetically modified bacteria able to consume and degrade methanol are valuable sources to decrease pollution and increase safe bioenergy production (Bruinsma et al., 2023).

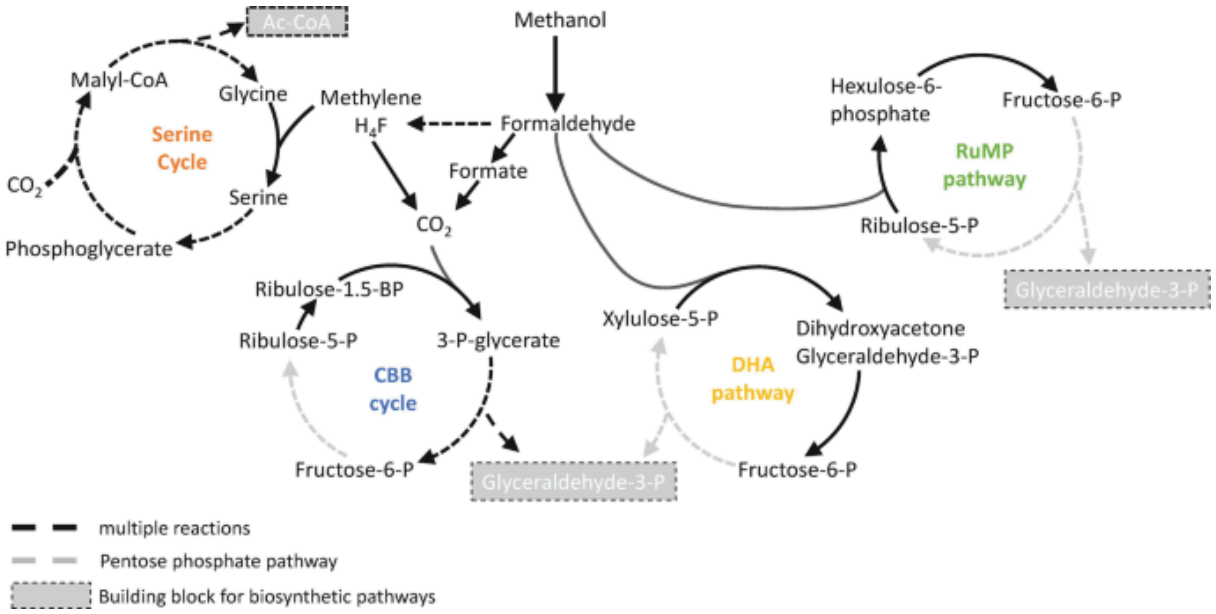

Figure 2. Aerobic degradation of methanol via serine cycle, Calvin-Benson-Bassham (CBB) cycle, dihydroxyacetone (DHA) cycle and ribulose monophosphate (RuMP). (adapted from Wendisch et al., 2021).

The utilization of methanol begins with oxidation to formaldehyde (Fig. 2) by the following methanol dehydrogenases (MDH): cytochrome c-dependent methanol dehydrogenase, which contains a pyrroloquinoline quinone cofactor (PQQ-MDH); NAD-dependent methanol dehydrogenase (NAD-MDH) and NADPH-dependent methanol dehydrogenase (NADPH-MDH) (Arfman et al., 1989). A highly toxic formaldehyde (due to its non-specific reactivity with proteins and nucleic acids) (Anthony, 1986; Sahm, 1977) can be degraded via 4 pathways: oxidative dissimilation to formate and CO_2 (Koopman et al., 2009), assimilation via the serine cycle (Chistoserdova et al., 2003), via dihydroxyacetone (DHA) cycle (Anthony, 1982), and assimilation via the ribulose monophosphate (RuMP) pathway (Kato et al., 2006).

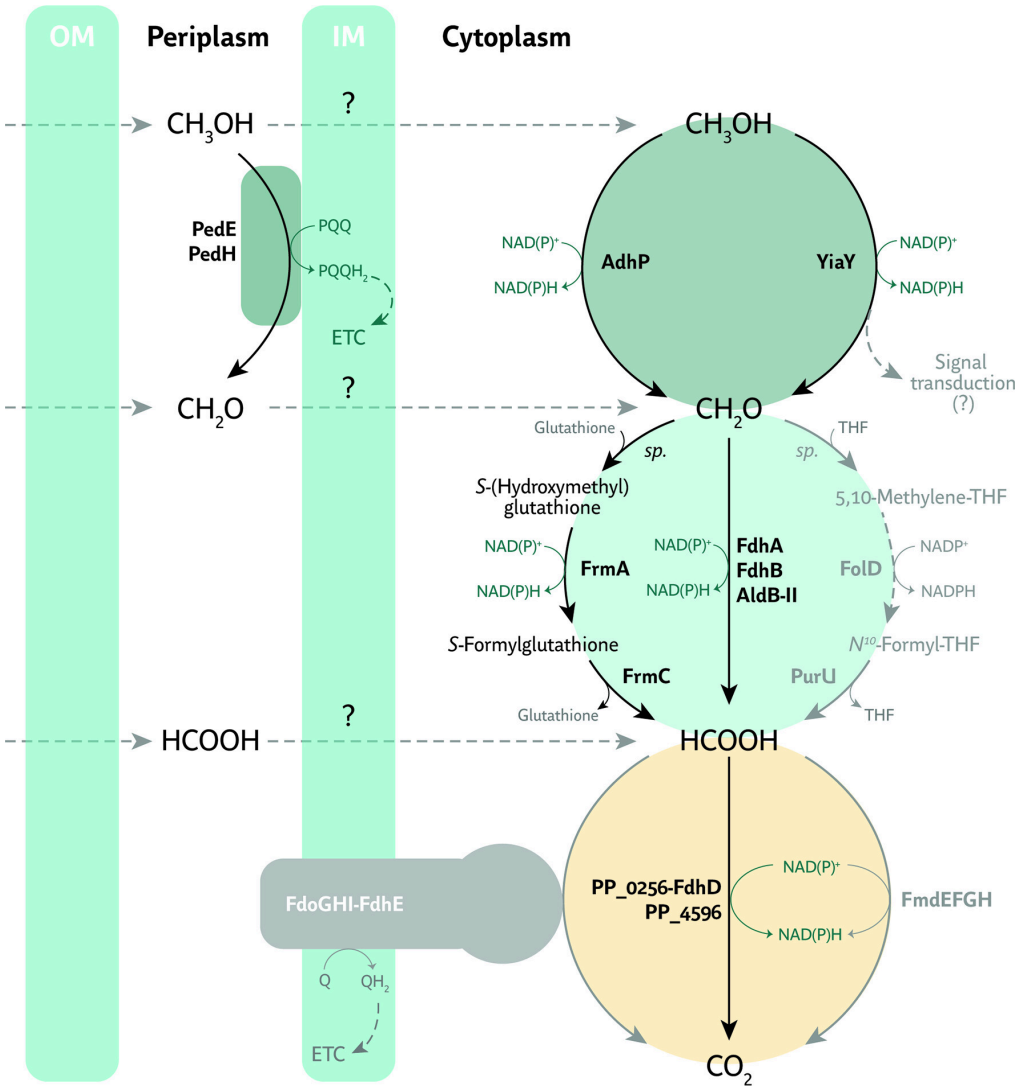

Figure 3. Model of the natural one-carbon metabolism pathways in *P. putida* updated. Formaldehyde (CH_2O), formate (HCOOH), and methanol (CH_3OH) can diffuse through the outer membrane (OM) or enter the bacterial periplasm through porines. The pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenases PedE and/or PedH in the periplasm can oxidize methanol, the most reduced form of these chemical species, to formaldehyde. Alternatively, methanol can pass through the inner membrane (IM) and be oxidized in the cytoplasm by other broad-substrate-range dehydrogenases, such as the NAD(P)+-dependent AdhP or YiaY proteins. The *ped* cluster may be transcriptionally activated by the latter by inducing a signal transduction cascade. Formaldehyde can then be converted to formate by a variety of routes, such as the tetrahydrofolate methylation loop or the glutathione-dependent (FrmAC) and thiol-independent (FdhA, FdhB, and AldB-II) processes. Finally, the NAD(P)+-dependent PP_0256-0257 and PP_4596 activities described in this work or, to a lesser extent, FdoGHI-FdhE and FmdEFGH, may totally oxidize formate to CO_2. One-carbon molecules are not yet known how they are transported across bacterial membranes (shown by a "?" symbol). For energy conservation, electrons collected by quinones (Q) or PQQ can be sent straight into the electron transport chain (ETC). (*sp.*) spontaneous reaction. (adapted from Turlin et al., 2023).

P. putida can degrade a broad spectrum of chemicals however the utilisation of C1 compounds is not part of its natural metabolism. A recent publication by Turlin et al., 2023

revealed that *P. putida* EM42 has several genes encoding alcohol dehydrogenases - PedEH (PQQ-MDH), AdhP and YiaY (both are NAD-MDH) (Turlin et al., 2023). The bacterium has also multiple formaldehyde (NAD-binding FdhAB; AldB-II; glutathione-dependent FrmAC) and formate (FDH; soluble NAD-dependent FDH FmdEFGH, membrane-bond FDH FdoGHI-FdhE cluster; thiol-independent FDH FdhD) dehydrogenases to detoxify formaldehyde and formate, respectively (Fig. 3). In methanol-exposed *P. putida* cells, the *pedE*, *pedH* and *aldB-II* were significantly overexpressed. They showed that the strain, where all alcohol dehydrogenases were knocked out, grew in a methanol-supplemented medium better than the parent strain revealing the toxic effect of formaldehyde in the metabolism of methanol. Overexpression of NAD+-dependent methanol dehydrogenase YiaY (which has been shown to act also as a transcriptional regulator (Bator et al., 2020)) in *P. putida* increased methanol uptake (Henson et al., 2021), while deletion of *yiaY* resulted in reduced toxicity to methanol due to the lowered expression of *ped* operon and *aldB-II* (Turlin et al., 2023).

1.3 Bacterial degradation of formaldehyde and formate

Lately, the C1 compounds have drawn interest as possible bacterial feedstock sources (Cotton et al., 2020). Despite the toxicity and disruptive effects, formate is considered to become one of the alternatives, as microorganisms are capable of degrading it, and use it as a renewable energy source for bioenergy and biofuel production in cell factories and bioreactors. Moreover, developing bioremediation strategies, which include the engineering of bacteria having the ability to degrade harsh chemicals into harmless products enzymatically, is the promising solution to globally emerging pollution (Bruinsma et al., 2023).

Aerobic growth on methanol and formate is supported by four primary metabolic pathways (Fig. 2): the Calvin-Benson-Bassham Cycle, the Ribulose Monophosphate (RuMP) Cycle, the Dihydroxyacetone (DHA) Cycle, and the Serine Cycle (Cotton et al., 2020). However, the Calvin Cycle has a relatively low energetic efficiency, ranging from 20% to 35%, due to its higher physiological reduction potential of NAD+ and high ATP consumption (Bennett et al., 2009).

The RuMP Cycle and DHA Cycle are two metabolic pathways used by bacteria for converting methanol into biomass. In both pathways, methanol is initially oxidized to formaldehyde, which is then condensed with pentose phosphate to produce metabolites that are reincorporated into the pentose phosphate pathway (PPP), regenerating the initial substrate and providing fixed carbon for cell growth. The RuMP Cycle demonstrates a higher energetic efficiency, ranging from 40% to 50%, compared to the DHA Cycle, which operates at a lower efficiency of 30% to 35% (Cotton et al., 2020).

The Serine Cycle represents an alternative metabolic pathway for converting methanol into biomass, where methanol is oxidized to formaldehyde by a pyrroloquinoline quinone (PQQ)-dependent enzyme, followed by the oxidation of formaldehyde to formate through the tetrahydromethanopterin system (Chistoserdova, 2011). However, these canonical oxidation routes could potentially be substituted with alternative mechanisms, such as a NAD-dependent enzyme or alternative formaldehyde oxidation pathways (Chistoserdova and Lidstrom, 2013).

The reductive glycine (rGly) pathway and the formolase pathway are synthetic, linear methanol and formate assimilation pathways (Fig. 4) that offer promising strategies for microbial metabolism engineering (Cotton et al., 2020). The rGly pathway involves the condensation of formate with tetrahydrofolate (THF) and subsequent reduction to 5,10-methylene-THF, which combines with CO_2 and ammonia to produce glycine (Cotton et al., 2020). This intermediate combines with CO_2 and ammonia to produce glycine, which is then deaminated to pyruvate for biomass synthesis (Bar-Even et al., 2013). Alternatively, serine can be assimilated into central metabolism via transamination, yielding hydroxypyruvate (Ishikawa et al., 1996) and 2-phosphoglycerate, or glycine can undergo deamination to glyoxylate, followed by self-condensation to produce tartronate semialdehyde, ultimately leading to glycerate and 2-phosphoglycerate (Cotton et al., 2020). Kim et al., 2020 showed that the *E. coli* strain introduced with rGly was able to produce biomass from formate and methanol. This route is theoretically most efficient in energy utilization, resource consumption, and biomass yield if compared for example with the Calvin cycle (Bar-Even et al., 2013). Kim et al., 2020 also proposed that replacing NAD-dependent methanol dehydrogenase with methanol oxidase could result in higher growth rates as this enzyme is faster and energetically more favourable.

The formolase pathway (Fig. 4) uses engineered enzymes to condense formaldehyde molecules into dihydroxyacetone, which enters central metabolism via phosphorylation to dihydroxyacetone phosphate (Siegel et al., 2015). This pathway shows potential for growth on methanol or formate by converting formaldehyde into dihydroxyacetone or glycolaldehyde (Poust et al., 2015; Lu et al., 2019), which can be oxidized to glyoxylate or converted to acetyl phosphate via phosphoketolase (Lu et al., 2019). However, the formolase pathway faces challenges due to poor enzyme kinetics (Lu et al., 2019).

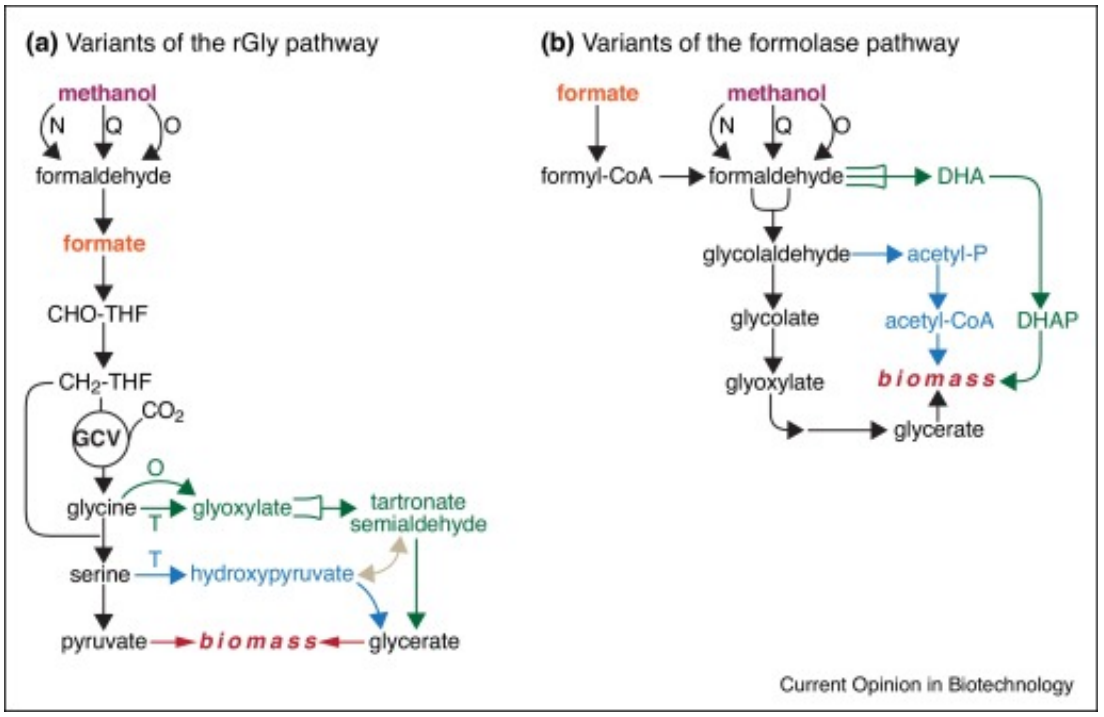

Figure 4. Synthetic pathways for the assimilation of formate and methanol, as well as structural variations. (a) The reductive glycine pathway's variations. An NAD-dependent ('N'), PQQ-dependent ('Q'), or O_2-dependent ('O') enzyme can oxidize methanol to formaldehyde. Blue arrows indicate the two possible assimilation pathways for glycine: conversion to serine and deamination to pyruvate or glycerate. A transaminase enzyme ('T') or an O_2-dependent oxidase enzyme ('O') can also convert glycine to glyoxylate. (b) Formolase pathway variants. An NAD-dependent ('N'), PQQ-dependent ('Q'), or O_2-dependent ('O') enzyme can oxidize methanol to formaldehyde. Formyl-CoA serves as an intermediary in the reduction of formate to formaldehyde. Formaldehyde can self-condense into glycolaldehyde or dihydroxyacetone (DHA, green arrows). Using native enzymes, this latter intermediate can be oxidized to glyoxylate, or it can be converted to acetyl phosphate using a repurposed phosphoketolase enzyme (Lu et al., 2019). (adapted from Cotton et al., 2020).

Pseudomonas putida KT2440 can tolerate up to 1.5 mM formaldehyde, and it has multiple formaldehyde dehydrogenases (Fig. 3), which may be constitutive (*fdhB*) or inducible (*rfmAB* operon), and convert highly toxic substrate to formate (Roca et al., 2009; Turlin et al., 2023). In *P. putida* EM42 formaldehyde was mostly degraded by a glutathione-dependent *frmAC* operon and glutathione-independent *fdhAB*, while at higher formaldehyde concentrations, AldB-II was responsible for detoxification. This can be explained by the enzyme's different formaldehyde affinities. The deletion of *frmAC* had the most significant effect on cells' methanol tolerance. (Turlin et al., 2023) While grown in methanol containing medium, the overexpression of *mdtABC/ompB* (encodes efflux transporter), oxalate/formate antiporter gene, and *cco-I* operon (encodes *cbb3*-type cytochrome oxidase) was detected in *P. putida* EM42 revealing the importance of active export in detoxification of aldehyde. At the same

time, several genes potentially involved in C1 metabolism were downregulated (Turlin et al., 2023).

In contrast to formaldehyde, microbes can tolerate over 100 mM formate, whose toxicity has been associated with acidification of the cytoplasm, impairment of the proton motive force, cause of oxidative stress, and inhibition of cytochrome *c* oxidases (Zaldivar and Ingram, 1999; Overkamp et al., 2002; Warnecke and Gill, 2005; Nicholls, 1975; Kirkpatrick et al., 2001). *Pseudomonas putida* also has two genuine formate dehydrogenases (FDHs), FdoGHI-FdhE and FmdEFGH (Zobel et al., 2016; Roca et al., 2009). *In vitro* enzymatic assays have demonstrated that *P. putida* KT2440 primarily metabolizes formate through the soluble, NAD+-dependent FDH FmdEFGH (Zobel et al., 2016). Turlin et al., 2023 showed that two soluble oxidoreductases of the broad FdhF/YdeP family and an accessory sulfurtransferase for formate dehydrogenase FdhD (Fig. 3) were significantly overexpressed in *P. putida* EM42 upon exposure to formate. This indicates that in *P. putida* oxidoreductases are involved in formate detoxification (Turlin et al., 2023) whereas FdhD is essential at sublethal concentrations of formate (Roca and Ramos, 2009).

The respiratory chain components encoding genes, *cioA* and *cioB* (oxidases of cytochrome *bd* family), were upregulated, and electron transport chain *nuo* operon (NADH-quinone oxidoreductase) was downregulated in response to the formate, pointing to a differential pattern of redox balancing. Also, the membrane-bound proton-pumping pyridine nucleotide transhydrogenase genes (*pntA* and *pntB),* involved in the regeneration of NADPH during the transfer of protons to the cell, were upregulated in the strain EM42 grown in formate-supplemented medium (Turlin et al., 2023).

1.4 Pseudomonas putida KT2440

Pseudomonas putida strain KT2440, classified as HV1 by the FDA (National Archives And Records Administration, 1982), is a popular choice for industrial biotechnology due to its robust redox metabolism, stress-resistance properties, rapid growth, and genetic accessibility. Its rapid growth, versatile metabolism, and nonpathogenic nature make it an attractive candidate for industrial biotechnology (Kampers et al., 2019).

The concept of a "chassis" in synthetic biology involves a well-defined genome and a manageable biochemical and regulatory network (Martínez‑García and De Lorenzo, 2024). Rational genomic deletions have been employed to optimize *P. putida* KT2440 for specific biotransformation purposes, but certain modifications may make it more susceptible to stresses (Lieder et al., 2015; Fan et al., 2020; Wynands et al., 2019; Espinosa et al., 2023;

Wang et al., 2015; Liang et al., 2020). Engineering *P. putida* KT2440 for anaerobic metabolism has faced challenges, necessitating additional genetic modifications and supplementation of external vitamins (Kampers et al., 2021). Strategies for controlling biofilm formation have been developed to enhance catalytic performance and bioconversion efficiency (Martínez‑García and De Lorenzo, 2024).

The strain's tolerance to toxic intermediates during bioprocesses, such as lignin processing and PET (polyethylene terephthalate) precursor production, makes it suitable for industrial applications (Kohlstedt et al., 2022; Erickson et al., 2022). Containment strategies have been proposed for engineered *P. putida* strains to mitigate environmental risks, such as engineering synthetic dependencies and recoding metabolic genes to prevent horizontal gene transfer (Asin-Garcia et al., 2021).

P. putida is a bacterium known for its efficient degradation of aromatic compounds through pathways like *ortho* and *meta* cleavage (Diaz, 2004; Harwood and Parales, 1996; Ornston, 1971), catalyzed by enzymes like catechol 1,2-dioxygenase and catechol 2,3-dioxygenase (Timmis et al., 1994). Plasmids in *P. putida* contain genes for the degradation of aromatic compounds, with the TOL plasmid being extensively studied for toluene degradation (Ramos et al., 1997; Nakazawa, 2002; Cases and de Lorenzo, 2005). Phenol degradation pathways, both *ortho* and *meta*, have been demonstrated in various strains, with regulatory systems involving transcriptional activators like XylR and DmpR (Ramos et al., 1997; Bertoni et al., 1998; Shingler et al., 1993). *P. putida*'s high NADPH regeneration, linked to glucose catabolism through the EDEMP cycle (Nikel et al., 2016), enhances its metabolic versatility. *P. putida* has potential in biotechnological applications, including the production of biopolymers like polyhydroxyalkanoate (PHA) (Prieto et al., 2015) and biosurfactants like rhamnolipids (Wittgens et al., 2011; Tiso et al., 2016). Engineered strains can valorize lignocellulosic biomass (Kohlstedt et al., 2018) into valuable products like *cis,cis*-muconate (Poblete-Castro et al., 2012; Lee et al., 2019; Xu et al., 2019). Mutagenesis in *P. putida* has revealed stress-induced transposition as a strategy for genetic adaptation under harsh conditions (Tegova et al., 2004). Biofilm development in *P. putida* involves proteins like LapA and LapF, which play crucial roles in biofilm initiation and maturation (Martínez-Gil et al., 2013; Duque et al., 2013; Moor et al., 2014).

Pseudomonads, including strains like *P. putida* KT2440, have evolved unique mechanisms to cope with environmental stressors, such as solvents and hydrophobic chemicals (de Bont, 1998; Sandoval and Papoutsakis, 2016). These stressors disrupt the cell envelope, affecting membrane functions and stability (Heipieper et al., 2007). One mechanism involves the

conversion of cis-unsaturated membrane fatty acids into their trans configuration, mediated by the periplasmic enzyme cis-trans-isomerase (Cti) (Heipieper et al., 2003; Heipieper et al., 1992). Efflux transporters (Godoy et al., 2010), particularly those of the resistance-nodulation-division (RND) family, play a critical role in conferring resistance to a broad spectrum of toxicants - antibiotics (de Bont and Kieboom, 2001; Puja et al., 2020), biocides (Chuanchuen et al., 2003), heavy metals (Blanco et al., 2016), mono- and polycyclic aromatics (Rojas et al., 2001; Yao et al., 2020; Henr´ıquez et al., 2020; Teran et al., 2006; Kieboom et al., 1998), short- and long-chain alcohols (Kieboom et al., 1998; Basler et al., 2018), (cyclo-)alkanes (Kieboom et al., 1998), monoterpenoids (Schempp et al., 2020), and aldehydes (Jayakody et al., 2018). Pseudomonads also employ strategies to prevent or repair damage caused by stressors, such as activating the SOS system (Maslowska et al., 2019) in response to DNA damage and up-regulating chaperones like GroEL/ES or ClpB (Hartl et al., 2011; Bosl et al., 2006). They also have a diverse set of redox enzymes that enable the conversion of harmful compounds into less noxious derivatives (Bitzenhofer et al., 2021).

Pseudomonas species offer a range of well-characterized expression systems, facilitating adjustable gene expression for various biotechnological purposes: these systems include inducible promoters with low leakiness and synthetic libraries of constitutive promoters (e.g. XylS/Pm, RhaRS/PrhaBAD, AntR/PantA, AraC/ParaB) (Martínez-García and de Lorenzo, 2017; Zobel et al., 2015; Calero et al., 2016).

1.4.1 Strain *Pseudomonas putida* KT2440_C1-S-Aux

Crucial components of microbial metabolism and biotechnological processes involve C1 substrates, such as formate and methanol (Bruinsma et al., 2023; Turlin et al., 2023). Microorganisms may develop and thrive in a variety of conditions thanks to these basic carbon molecules, which act as alternate carbon sources for them (Bruinsma et al., 2023). Further to being sustainable feedstocks for bioproduction processes, C1 substrates can be obtained from renewable resources. (Bruinsma et al., 2023).

The strain *Pseudomonas putida* KT2440_C1-S-Aux (CSA) was engineered as a potentially promising option to fulfill the requirements of a modified strain, capable of utilizing the harsh chemicals, like methanol and formate, as a feedstock, and outperform the existing variants in durability in conditions, harmful for previously proposed organisms (Bruinsma et al., 2023).

The reductive glycine pathway (rGlyP) has been proposed as a substitute mechanism for C1-assimilation in model microorganisms in recent years. Prior to its discovery in nature as a CO_2 and formate assimilation system, this linear pathway was created as a synthetic pathway. (Bar-Even et al., 2013; Cotton et al., 2018; Figueroa et al., 2017; Lowe and Kremling, 2021; Sanchez-Andrea et al., 2020).

To establish the rGlyP, the pathway was divided into three distinct modules: C1, C2, and C3 (Fig. 5). The C1 module is responsible for converting formate into 5,10-methylene-THF. This compound then undergoes further conversion into glycine through the reverse function of the glycine cleavage system (GCS) within the C2 module. Subsequently, in the C3 module, glycine is combined with another molecule of 5,10-methylene-THF to form serine. Ultimately, serine can be transformed into pyruvate, which serves as a precursor to biomass production (Bruinsma et al., 2023).

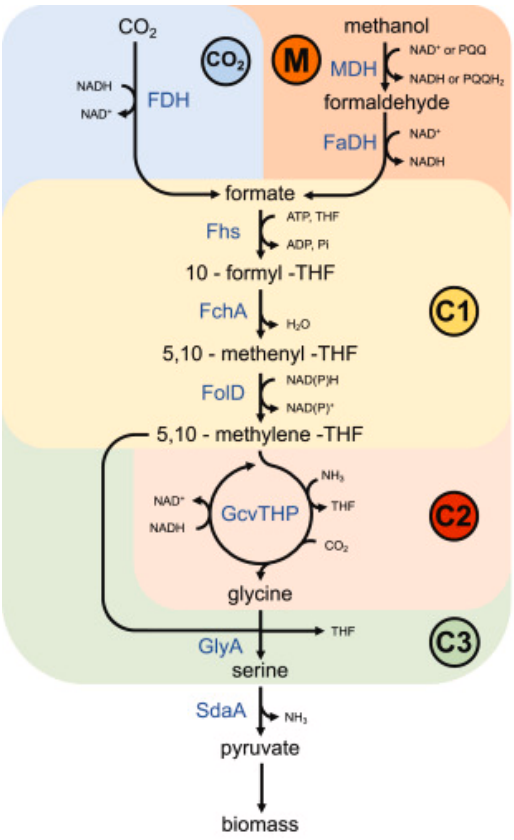

Figure 5. The modules of the reductive glycine pathway (rGlyP) for the metabolism of methanol and CO_2. Methanol and CO_2 are converted to formate, respectively, via the CO_2 and M modules. The C1 module then converts formate to 5,10-methylene-THF. The C2 module, which contains the reverse glycine cleavage machinery, then converts the 5,10-methylene-THF to glycine. Serine is created in the C3 module by condensing glycine with 5,10-methylene-THF. Subsequently, serine is converted to pyruvate, which gives the cell the biomass it requires. The following abbreviations stand for: formate dehydrogenase (FDH), methanol dehydrogenase MDH), formaldehyde dehydrogenase (FaDH), formate THF-ligase (Fhs), formyltetrahydrofolate cyclohydrolase (FchA), bifunctional methylenetetrahydrofolate dehydrogenase/methyltetrahydrofolate cyclohydrolase, (FolD), glycine cleavage system (GcvTHP), serine hydroxymethyltransferase (GlyA), serine deaminase (SdaA), and tetrahydrofolate (THF). (adapted from Bruinsma et al., 2023).

To scrutinize the functionality of the C1 and C3 modules, a strain termed C1-S-Aux was devised, involving the deletion of genes of both GCSs (Δ*gcvTPH-I/II*) and the D-3-phosphoglycerate dehydrogenases (Δ*serA*/Δ*PP_2533*) (Bruinsma et al., 2023), which are crucial for serine and C1 precursor molecules production. Consequently, this strain could not synthesize essential components required for various biosynthetic processes, including purines, thymidine, coenzyme A, and methionine (Yishai et al., 2017). As a result, growth on conventional carbon sources like glucose could only be restored through the supplementation of serine (Yishai et al., 2018) or by ensuring the presence and activity of both C1 and C3 modules, utilizing glycine and formate as substrates (Fig. 6) (Bruinsma et al., 2023).

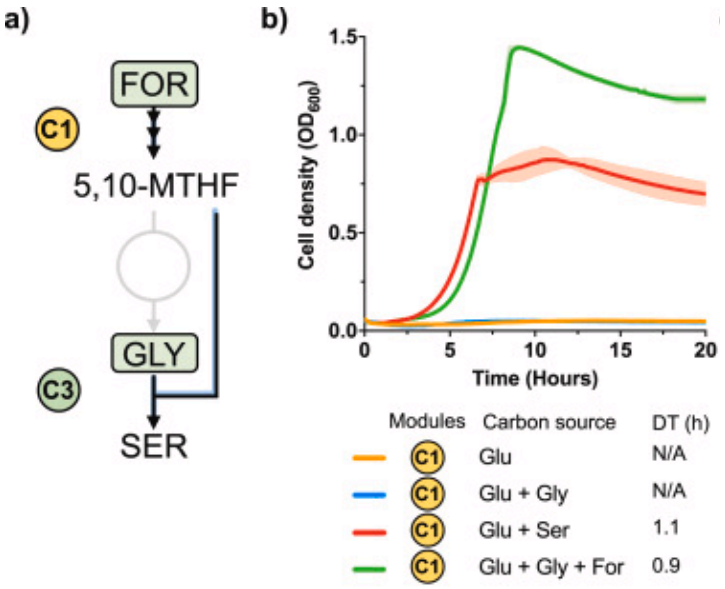

Figure 6. Formate-dependent growth of the C1-S-Aux possessing C1 and C3 modules (a) is possible when glycine is present (b). Growth in the glucose-containing medium is restored if serine is added (b) Abbreviations: (Gly), glycine, (Ser), serine, (For), formate, (5,10-MTHF), 5,10-methylene-THF, (Glu), glucose, (N/A), not applicable. (adapted from Bruinsma et al., 2023).

1.4.2 Strain *Pseudomonas putida* KT2440_C1-S-Aux_pM

After the successful construction of *Pseudomonas putida* KT2440_C1-S-Aux Bruinsma et al., 2023 went further and constructed the strain KT2440_C1-S-Aux_pM (CSA_pM) able to assimilate methanol (Fig. 7a). To achieve this, the pM plasmid was constructed by introducing methanol dehydrogenase (MDH) and NAD-dependent formaldehyde dehydrogenase *fdhA* in the pC1 vector (Bruinsma et al., 2023). Notably, when no MDH gene was expressed (the M-module was absent), no growth on glucose supplemented with methanol and glycine was observed in the C1–S-Aux strain (Fig. 7b) (Bruinsma et al., 2023). Distinct growth patterns were observed while different MDHs from various bacteria were introduced, indicating variations in their efficiency and supporting bacterial growth. After testing they selected the MDH of *Cupriavidus necator* as with it the shortest lag phase was obtained. The pM_*C.necator* (pMnec) plasmid contains the genes *mdh* CT4-1 from *Cupriavidus necator*, *fdhA* from *Pseudomonas putida*, and *fhs*, *fchA*, and *folD* from *Clostridium ljungdahlii* (Bruinsma et al., 2023).

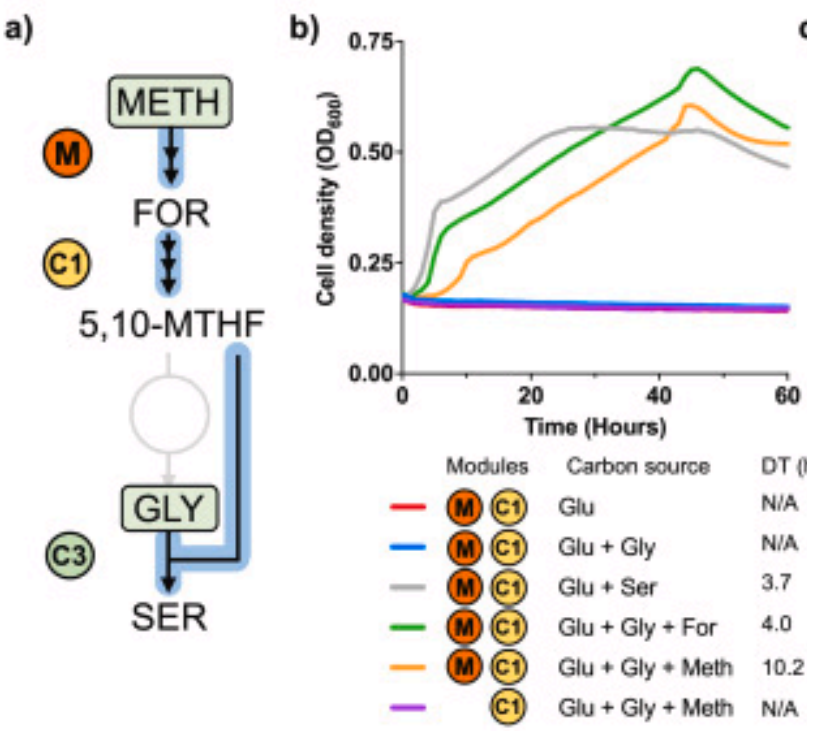

Figure 7. Methanol-dependent growth of C1–S-Aux is achieved when the M, C1, and C3 modules are present (a). Growth of the strain in the glucose-containing medium is observed only when serine or formate and glycine or methanol and glycine are present (b). Growth in methanol+glycine medium is not possible if M module is missing Abbreviations: (5,10-MTHF), 5,10-methylene-THF, (Glu), glucose, (N/A), not applicable, (Meth), methanol, (For), formate, (Gly), glycine, (Ser), serine. (adapted from Bruinsma et al., 2023).

As methanol oxidation is the major bottleneck of the process it has been suggested to use instead of the NAD-dependent MDHs thermodynamically more efficient PQQ-MDHs (Kim et al., 2020). Bruinsma et al., 2023 used also *P. putida* PQQ-MDH, but they concluded that further optimization is needed to achieve similar growth parameters as with *C. necator* MDH.

2. EXPERIMENTAL PART

2.1 Aims of the study

Methanol and formate as C1 substrates can be used as alternative microbial feedstocks for the biobased production of chemicals and fuels. Unfortunately, aerobic cell factory organisms, such as *Pseudomonas putida* can not naturally use the C1 substrates. However, by using synthetic biology tools the methanol and formate assimilating strains have been constructed. As C1 compounds are harmful to bacteria, we need to know if the growth conditions cause stress and how to monitor different stress responses in the cells.

How studied strains, *P. putida* strain KT2440 and genetically modified strains CSA and CSA_pM, are growing in C1 compounds, and are these substrates and growth conditions stressful, the following aims were outlined:

- Compare the stress response of KT2440 and C1 compounds utilizing strains CSA and CSA_pM;
- test stress response reporter systems pAG032 and pBSibp_Amp;
- analyse the growth and relative fluorescence dynamics while strains are growing in C1-supplemented LB or minimal medium.

2.2 Materials and Methods

2.2.1 Bacterial strains, plasmids and culture conditions

The following bacterial strains were used in the experiments: *Pseudomonas putida* KT2440 (in the following paragraphs, we will use the abbreviation KT2440); *Pseudomonas putida* KT2440_C1-S-Aux (in the following paragraphs, we will use the abbreviation CSA) and *Pseudomonas putida* KT2440_C1-S-Aux_pM_*C.necator* (in the following paragraphs, we will use the abbreviation CSA_pMnec) (Table 2).

Table 2. Strains and plasmids.

strain/plasmid	abbreviation	description	reference
Pseudomonas putida KT2440	KT2440	Wild-type strain; mt-2 derivative cured of the TOL plasmid pWW0	Bruinsma et al., 2023
Pseudomonas putida KT2440_C1-S-Aux	CSA	KT2440 Δ*gcv*TPH-I Δ*gcv*TPH-II Δ*serA* ΔPP_2533: Auxotrophic for C1 moieties and serine when grown on glucose.	Bruinsma et al., 2023
Pseudomonas putida KT2440_C1-S-Aux _pMnecator	CSA_pMnec	Strain KT2440 Δ*gcv*TPH-I Δ*gcv*TPH-II Δ*serA* ΔPP_2533: with plasmid pMnec (pSEVAb24 containing *mdh* CT4-1 from *Cupriavidus necator*, *fdhA* from *Pseudomonas putida* and *fhs*, *fchA* and *folD* genes from *Clostridium ljungdahlii* DSM 13528 :ori (pRO1600/ColE1); Km^R)	Bruinsma et al., 2023
Pseudomonas putida KT2440_pBSibp_ Amp	NC	Negative control for HS	Current work
pAG032	pAG032	pAG001 plasmid with *rfp* under control of *XylS/Pm*, *cfp* under control of *ibpfxs* and *yfp* under control of *rpsJ;* Amp^R	Gawin et al., 2019
pBSibp_Amp	pBSibp	Plasmid is a medium-copy number pBRR1-based promoter probe vector pBLKT, containing ampicillin resistance gene and *rfp* (mScarlet-I) under control of *ibpfxs*	Gift from Riho Teras

The following media were used for the growth of the bacteria:

1) Luria Bertani (LB) medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl);
2) Minimal liquid medium contained 1xM9 (KH_2PO_4, 3 g/l; NaCl, 0.5 g/l; Na_2HPO_4, 7 g/l; NH_4Cl, 1 g/l), microelements solution (667 µM MgO, 50 µM $CaCO_3$, 40 µM $FeSO_4$, 12.5 µM $ZnSO_4$, 12.5 µM $MnSO_4$, 2.5 µM $CuSO_4$, 2.5 µM $CoSO_4$, 1.9 µM H_3BO_4), 0.2% glucose, 0.2% casamino acids (CAA; acidic hydrolysate of casein), tryptophane (1 mg/ml) and 2 mM L-serine.

To obtain solid media, agar was added at a final concentration 15 g/L. Antibiotics, Kanamycin (Km; 50 μg/ml) and Benzylpenicillin (Bp; 1 mg/ml) were used for the selection of plasmids and bacterial strains.

2.2.2 Preparation of competent cells, electroporation

300 μl of overnight grown *P. putida* KT2440 cells were transferred to the 1.5 ml Eppendorf tube, after centrifugation (1 min; 12 000 rpm) the supernatant was discarded. The cell pellet was washed three times with 1 ml 0.3 M saccharose solution. After washing, cells were resuspended in 200 μl of 0.3 M saccharose. A solution of 50 μl of the competent cells and 0.5 μl plasmid of interest was transferred into the sterilized electroporation cuvette. The electroporation was carried out with "BioRad" electroporator at 2500V. Then, 2 ml of LB medium was immediately added to the cuvette, and after fast resuspending, the solution was poured back into the glass tube. The cells were incubated at 30 °C for 1h in a rotary shaker (180 rpm).

After incubation, the cell culture was centrifuged (3 min; 8000 rpm), and approximately 100 μl of medium was left to suspend the cells. The cell suspension was plated onto LB-Bp (in the case of the strains KT2440 and CSA) or LB-Km/Bp (strain CSA_pMnec) selective plates and were incubated at 30 °C. 30 colonies from each overnight selective plate were randomly chosen and inoculated into a new selective plate. Then, the heat-shock response (paragraph 2.2.4) was performed to select clones with correctly inserted plasmids.

For the preparation of overnight cultures, the strains were inoculated into a liquid medium, either LB or Minimal supplemented with respective antibiotic(s), and tubes were incubated on the rotary shaker (30 °C; 180 rpm).

2.2.3 Determination of growth parameters and chemical-induced stress response

The growth of the bacteria was measured in 96-well Greiner Black plates (black plate, clear bottom, clear lid) containing 150 μl LB and Minimal media supplemented with methanol (5%), formate (30 mM), or ciprofloxacin (0.04 μg/ml). The absorbance of overnight cultures at 580 nm was measured with "BioChrom ULTROSPEC 7500", and the amount of cell suspension was calculated to obtain after reinoculation in each well A_{580} 0.1. From eight wells with one medium seven were inoculated with respective strains and one was left for abiotic control (without cells). The growth of the bacteria was recorded with a thermostated (30 °C) "BioTek SYNERGY|H1" microplate reader at 580 nm, in YFP (mVenus): 505 nm excitation wavelength, 540 emission wavelength, gain 50 and in RFP (mScarlet-I): 570 nm excitation

wavelength, 600 emission wavelength, gain 50 for determination of chemical mediated stress response.

2.2.4 Determination of the Heat-shock response in bacteria

The overnight culture, taken approximately 100-200 µl, was divided into two parallels (30 °C and 42 °C) in 5 ml of a new liquid medium and antibiotics were added. Then, the tubes were placed to the rotary shaker (30 °C; 180 rpm) for 2-3 hours. After that, cultures were separated to 30 °C and 42 °C and left to grow 2 hours more (OD approx. 0.5). After that 100 µl of the sample (4 technical parallels) was transferred to the 96-well Greiner Black plates (black plate, clear bottom, clear lid) and the measurements were done with "TECAN Infinite M200 PRO" in 580 nm for growth, in CFP (mCerulean) for pAG032 strains: 433 nm excitation wavelength, 475 nm emission wavelength, 9 nm excitation bandwidth, 20 nm emission bandwidth, gain 80, integration time 20 µs and Z-Position 20000 µm; and RFP (mScarlet-I) for pBSibp_Amp strains: 570 nm excitation wavelength, 600 nm emission wavelength, 9 nm excitation bandwidth, 20 nm emission bandwidth, gain 80, integration time 20 µs and Z-Position 20900 µm.

2.2.5 PCR

PCR was applied for the verification of the presence of pMnecator plasmid in CSA_pMnec (Bruinsma et al., 2023) strain. The total volume of PCR mixture was 25 µl, including water, 10xDreamTaq Green buffer (Thermo Fisher Scientific, USA), 20 mM $MgCl_2$, 200 µM concentrations of each dNTPs (dATP, dGTP, dCTP, dTTP), 0.5 U thermostable DreamTaq DNA polymerase (Thermo Fisher Scientific, USA), primers (10 mM), bacterial cells. The primers pM_KF (5'-CAA GAT CGT GGT GGG CTA CA-3') and pM_KR (5'-GTC GTA GGC GAA CTG GAA GT-3') were used for amplification of plasmid. Bacterial cells, taken with a pipette tip, were mixed with 25 µl of water and put at 95 °C for 15 minutes to lyse before the reaction. The reaction was performed in the Eppendorf Mastercycler PCR machine. PCR conditions were as follows: denaturation at 95 °C for 1 min, annealing of primers at 57 °C for 45 s, synthesis of DNA at 72 °C for 1 min, product size approximately 660 bp. In order to amplify a sufficient amount of DNA molecules cycles were repeated 32 times. The program ended with the final extension step, 72 °C for 10 min.

2.2.6 Gel electrophoresis

Gel electrophoresis was used to analyse the conducted PCR reactions. PCR probes (5 µl) were loaded to the agarose gel (1%) containing fluorescent dye ethidium bromide (0.5 µg/ ml). A GeneRuler 1kb DNA ladder (3.5 µl) (Thermo Fischer Scientific, USA) was added to one line

of the gel for determination of the product size. The electrophoresis was performed at 100 V for 17 min in 1xTAE (50 mM Tris-acetate; 1 mM EDTA; pH 8.2) buffer. The resulting gel was visualized under UV light to assess the presence of bands and their sizes.

3. RESULTS AND DISCUSSION

3.1 Heat shock response

Currently, the interest in bacterial heat-induced stress response is mostly driven by the demand for sustainable resources for enzyme, protein, and biofuel production on industrial scale. Understanding the mechanisms that naturally regulate the thermotolerance of microorganisms may lead to the engineering of resistant bacteria (Bruinsma et al., 2023) and effective manufacturing, increasing performance and safety in industrial fermentation processes, where high temperatures can hinder bacterial growth and productivity.

The heat shock response experiment was conducted in order to observe how different bacterial strains react to heat-induced stress by means of activation of σ^{32} (which is encoded by *rpoH* gene) and σ^{E}, which initiates the transcription of the *rpoH* (Alba and Gross, 2004).

The experiment was performed using wild-type strains: KT2440, CSA, CSA_pMnec (Bruinsma et al., 2023), and the same strains transformed with pAG032 plasmid (Gawin et al., 2019) or with pBSibp_Amp plasmid (Table 2).

3.1.1 HS response with pAG032 carrying strains

Plasmid-based reporter systems are useful tools to monitor stress responses. Gawin et al., 2019 constructed a plasmid pAG032 to follow the HS response in bacteria. Expression of *cfp* on the plasmid relies upon the σ^{32}-dependent P*ibpfxs* tandem promoter which responds to heat shock and accumulation of misfolded proteins.

The plasmid pAG032 was transformed into strains and cells were grown on 30 and 42 °C. After 2 or 3 h growth the fluorescence of CFP was measured, and heat shock response was presented in relative fluorescence units (rCFP; fluorescence values were divided with absorbance values at 580 nm). The HS experiments were performed in LB (Fig. 8A) and minimal medium (contains glucose, cas amino acids, serine, and tryptophan) (Fig. 8B). The data obtained after 2 and 3 h incubations were quite similar in both media. Gawin et al., 2019 showed that in *P. putida* the rCFP increased already after 30 min and reached the max value after 1.5 h.

As can be seen from Figure 8 high rCFP values were obtained in LB medium at 30 °C. This may be explained by the fact that some species from the genus *Pseudomona*s, including KT2440, are producing fluorescent pigments (for example siderophores) that have overlapping fluorescence spectra (405/460; Dell'Anno et al., 2022) with mCerulean (CFP; 433/475). It has been shown that siderophore production depends on a medium composition,

for example from the concentration of sulphate, iron, and magnesium ions (King et al., 1948) and as in the LB medium these are lower than in minimal medium the cells are producing more pigment.

With the vector system pAG032, the values of rCFP in the minimal medium at the optimal growth temperature of the strains were two times smaller than in the LB medium (Fig. 8). The most fluctuating results gave strain CSA_pAG032, which even at 30 °C formed clumps, and therefore the spectrometric measurements vary more.

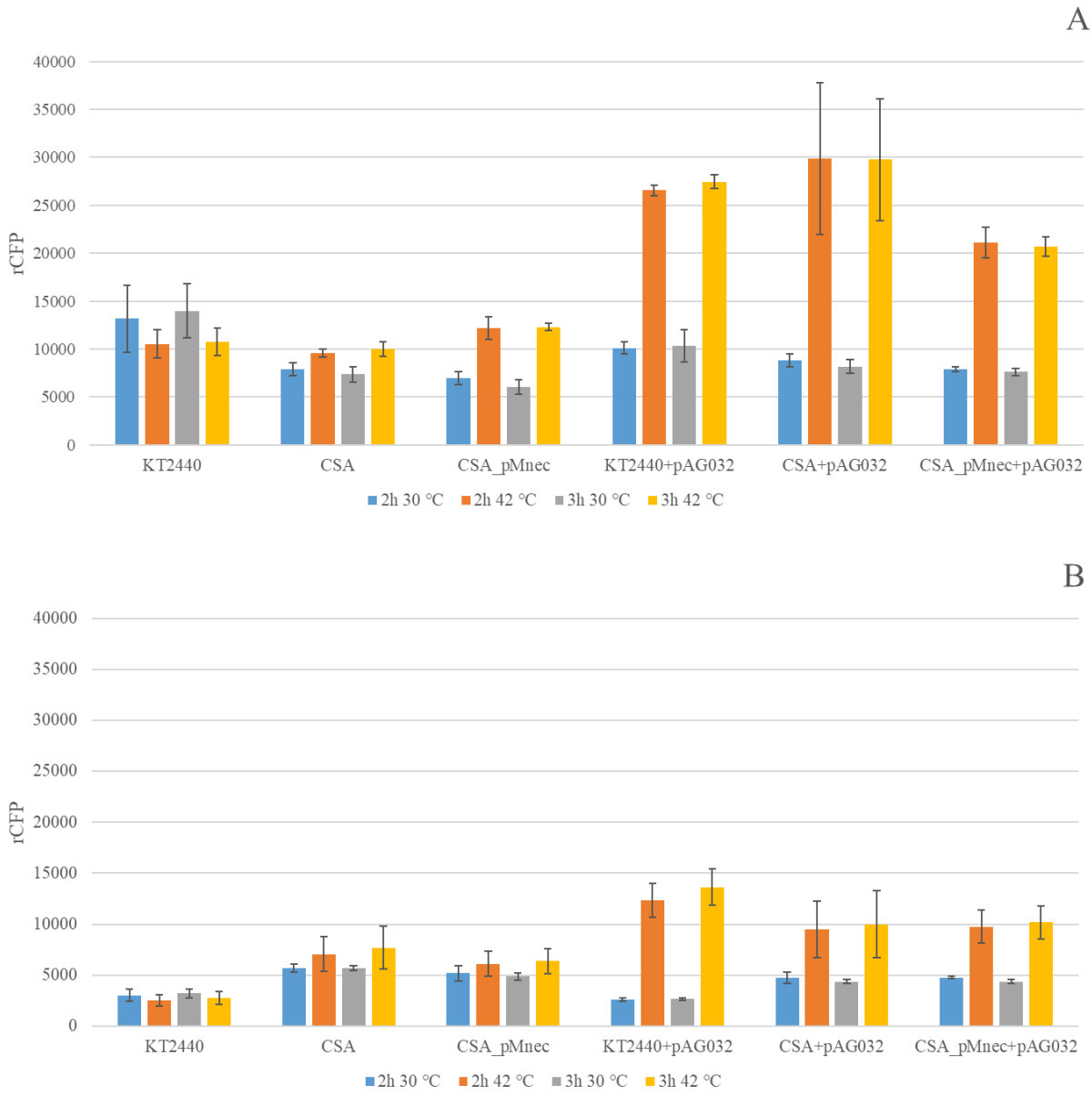

Figure 8. Average relative blue fluorescence (rCFP) of wild-type strains (KT2440, CSA and CSA_pMnec), and transformed strains with pAG032 plasmid grown in LB (A) and minimal (B) medium for 2 and 3 h at 30 °C and 42 °C. Averages of at least three biological parallels with standard deviations are shown.

Calculation of ratios of relative fluorescences at 42 and 30 °C show that the heat-induced response is relatively small, in LB medium it was 2.7-3.5 (Fig. 9A) and in minimal medium 2.0-5.0 (Fig. 9B). These data are in accordance with the result of Gawin et al., 2019 who obtained with *P. putida* KT2440 in a minimal medium 5.9-fold increase of *cfp* expression from P*ibpfxs* after shifting cultures to 42 °C. It is interesting that the heat shock response of three strains in the minimal medium is so different (Fig. 9B). The highest values were obtained with the strain KT2440_pAG032 while genetically modified strains CSA and CSA_pMnec had more than two times smaller differences (Fig. 9B). At the same time, the ratios of rCFPs of wt strains (without pAG032) in this medium were around 1 confirming that the increase of rCFP of the plasmid-carrying strains is caused by heat shock (Fig. 9B). But in the LB medium grown wt engineered strains had higher differences than KT2440 (Fig. 9A). This potentially could be explained by the introduction of reductive glycine pathway (rGlyP) into the strains, which causes additional load on metabolism and generally growth, considered a metabolic burden.

Analysing the results presented in Figures 8 and 9 we can conclude that plasmid pAG032 can be used for the measurement of heat shock response as higher rCFP values were obtained with plasmid-carrying strains after incubation at higher temperatures. Still, due to the high background fluorescence of wt strains, caused probably by the synthesis of the fluorescent pigments, we decided to use a plasmid where the P*ibpfxs* promoter controls the expression of the *rfp* gene encoding the red fluorescence-producing protein.

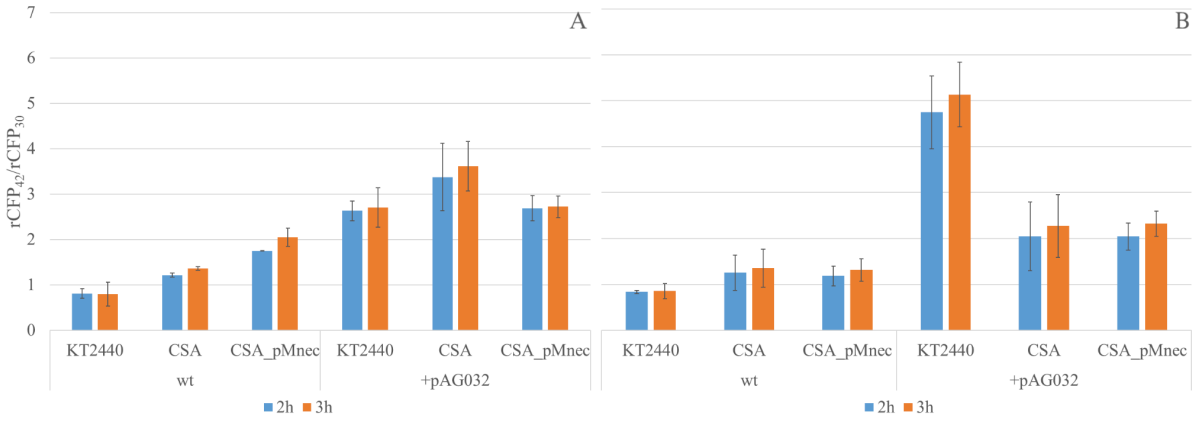

Figure 9. The ratio of relative fluorescences ($rCFP_{42}/rCFP_{30}$) of wild-type strains (KT2440, CSA and CSA_pMnec), and transformed strains with pAG032 plasmid grown in LB (A) and minimal (B) medium for 2 and 3h. Averages of at least three biological parallels with standard deviations are shown.

3.1.2 HS response with pBSibp_Amp (pBSibp) carrying strains

The plasmid pBSibp_Amp (abbreviation pBSibp), was constructed specifically to monitor the HS response in bacteria (Table 2). This plasmid encodes the ampicillin resistance gene and *rfp* (mScarlet-I (Bindels et al., 2017); 570/600) expression is under the control of σ^{32}-dependent P*ibpfxs* promoter (Gawin et al., 2019), which allows measuring RFP directly, as in pAG032 plasmid CFP expression. The *rfp* was chosen as the natural fluorescence of pseudomonads does not affect the measurement of RFP fluorescence (Loeschcke and Thies, 2015).

The experiments were performed as in 3.1.1. Additional negative control (NC) - strain KT2440 with an inserted plasmid pBSibp that has an ampicillin resistance but lacks *rfp* expression at higher temperatures - was included to follow the effect of the introduced plasmid. Wild-type strains served as negative controls to verify the functioning of the constructed reporter system.

The relative RFP values of wt strains and NC at both incubation conditions are low, while strains with transformed pBSibp have high values at 42 °C (Fig. 10) revealing that expression of *rfp* from the constructed plasmid is due to the heat-induced shock.

The rRFP values of strains with plasmid pBSibp are slightly higher at 30 °C than strains without the plasmid, indicating probably the burden of bearing the plasmid. Additionally, in the minimal medium, the heat-induced stress response is reduced in comparison of LB grown cells (Fig. 10), and also the rRFP of KT2440_pBSibp has higher values than CSA_pBSibp and CSA_pMnec_pBSibp (Fig. 10A). Performing the experiments we noticed that although serine was added to the minimal medium to support the growth of engineered auxotrophic strains, the growth of these strains was slower than KT2440_pBSibp.

The ratios of the relative fluorescences indicate that strains grown in LB (Fig. 11A) generally give higher values than in minimal medium (Fig. 11B). These results of our experiments agree with the results of Mason et al., 1999, where it was pointed out that in the complex medium, the heat-shock-induced response may be higher due to the presence of high concentrations of different compounds (amino acids, proteins, etc.) that can potentially be damaged by exposure to higher temperatures and cause additional stress for bacteria. The strain KT2440 transformed with pBSibp plasmid, growing in LB medium, exhibits the highest, 16-fold increase after 2 h of heat shock experiment and 18-fold increase after 3 h time point.

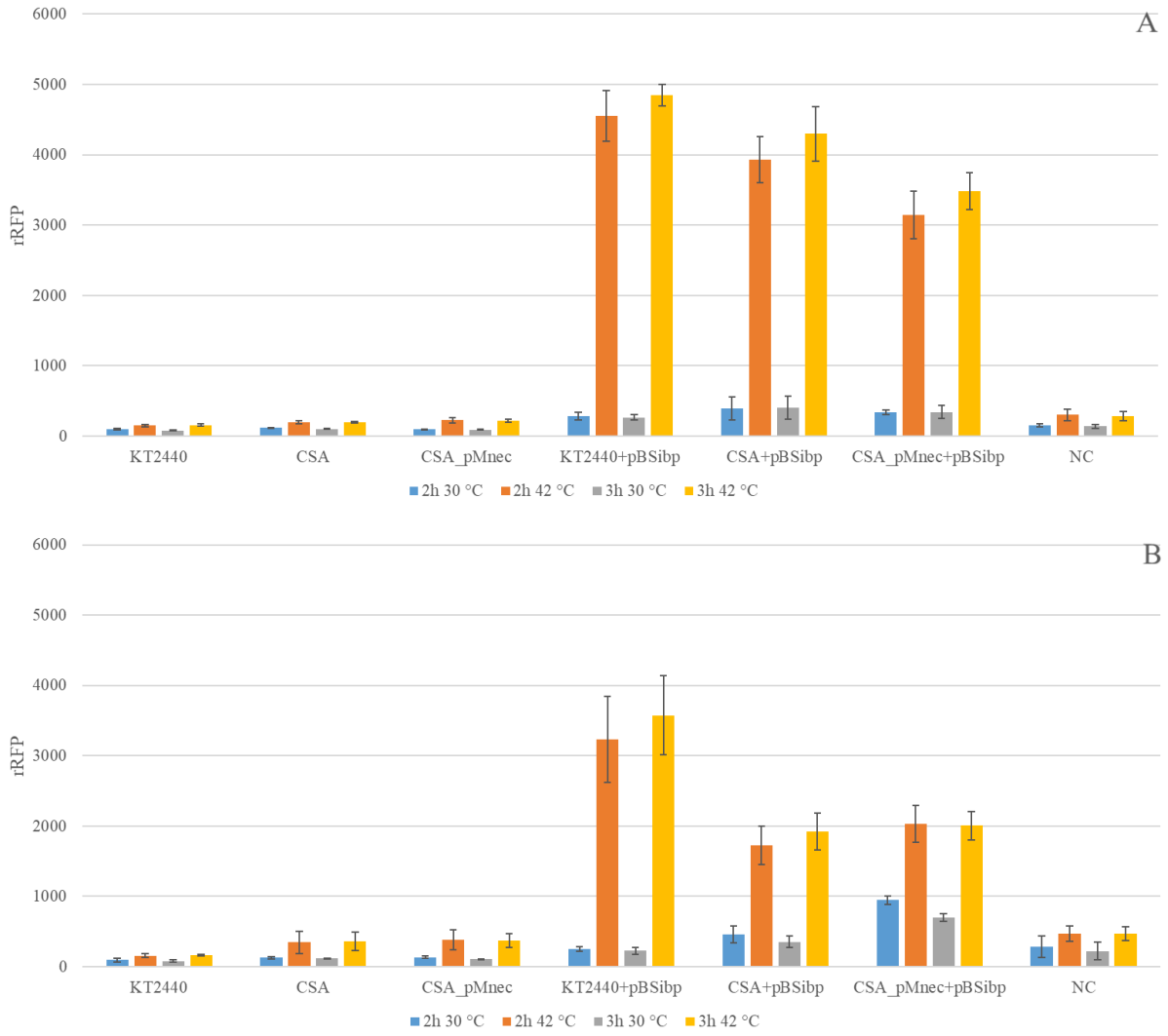

Figure 10. Average relative red fluorescence (rRFP) of wild-type strains (KT2440, CSA and CSA_pMnec), and transformed strains with pBSibp plasmid grown in LB (A) and minimal (B) medium for 2 and 3 h at 30 °C and 42 °C. NC - strain KT2440 with an inserted plasmid pBSibp; is ampicillin resistant but *rfp* is not expressed at elevated temperatures. Averages of at least three biological parallels with standard deviations are shown.

Moreover, while growing in minimal medium, the same strain establishes the highest increase of ratio as well, being an almost 16-fold increase at a 3 h time point. The expression of *htpG* (encodes heat shock protein) in glucose mineral medium grown *E. coli* strain was not affected or was even repressed in heat stress conditions (Mason et al., 1999). The reduced expression of *rfp* (Fig. 10B) and accordingly lower ratios (Fig. 11B) were detected also with engineered strains CSA and CSA_pMnec in the minimal medium that may be explained with the used medium and/or with the introduction of rGlyP pathway, which is not natural to *P. putida* and may cause an additional metabolic burden in stress conditions.

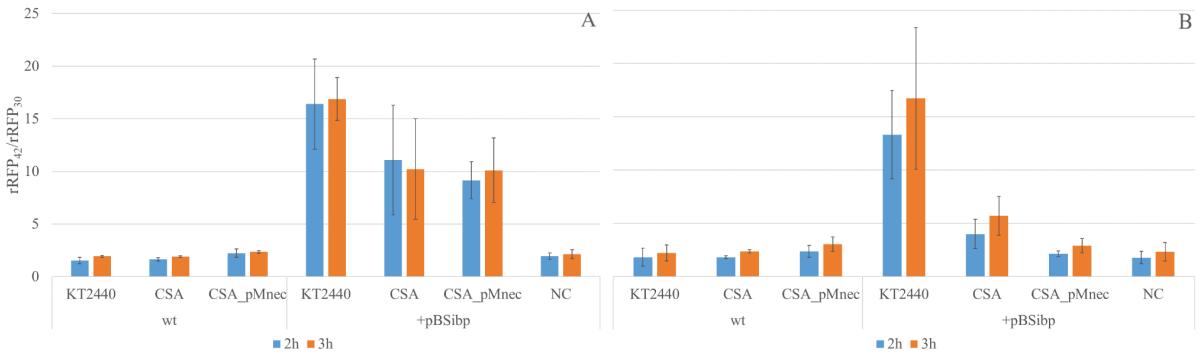

Figure 11. The ratio of relative fluorescences ($rRFP_{42}/rRFP_{30}$) of wild-type strains (KT2440, CSA and CSA_pMnec), and transformed strains with pBSibp plasmid grown in LB (A) and minimal (B) medium for 2 and 3h. Averages of at least three biological parallels with standard deviations are shown.

To sum up, the pAG032 plasmid is a very progressive and convenient stress response reporter system, as it allows for simultaneous measurements of a wide range of stress responses. However, it is unsuitable for measuring heat-induced stress in *P. putida* due to their natural fluorescence proteins' and CFP overlapping fluorescence spectra.

The specifically engineered pBSibp plasmid to overcome the fluorescence overlap issue of pAG032 is indeed working, giving significantly higher ratios of relative fluorescence and, consequently, the stress response. The main disadvantage of these plasmids is their inability to measure genuine heat-induced stress response, as along with heat shock proteins, the ppGpp-mediated stress response may also be activated in cases of a sudden temperature elevation (Schäfer et al., 2020).

In the next paragraphs, we conducted continuous monitoring of bacterial growth to examine the temporal dynamics of stress response in *P. putida* strains. By employing techniques such as optical density measurements, we aim to track changes in bacterial growth parameters over time. This approach will provide valuable insights into how *Pseudomonas* adapts to stressors across different phases of growth.

3.2 Chemical-induced stress response

The detrimental consequences of exposure to dangerous substances, such as antibiotics, heavy metals, and metabolic inhibitors, are referred to as "chemical stress" in bacteria. Numerous adaptive reactions can result from these stresses' disruption of physiological functions, such as protein synthesis, membrane integrity, and DNA replication (Aertsen and Michiels, 2005). Under chemically induced stress circumstances, the ppGpp-mediated stress response plays a crucial role in the survival of bacteria (Potrykus and Cashel, 2008).

The vitality of the (p)ppGpp (guanosine tetraphosphate or guanosine pentaphosphate) stress response underlies the fact that it is considered to be one of the main mechanisms responsible for adaptation to stressful conditions of microorganisms, including the major changes in gene expression and metabolic pathways, to ensure bacterial survival and proliferation (Roghanian et al., 2019). Understanding the role of alarmone molecule ppGpp in chemical- or starvation-induced stress response will lead to the optimization of biotechnological processes, by the creation of innovative and more durable microbial strains (Bruinsma et al., 2023), or, additionally, the development of new therapeutic strategies targeting microbial virulence, as this is the part of the function of ppGpp stress response as well (Ahmad et al., 2019).

ppGpp is known for its ability to influence bacterial physiology and confer antibiotic tolerance (Dalebroux et al., 2010). Fundamentally, (p)ppGpp is important for the regulation of metabolic balance during adaptation and exponential growth phases, preventing excessive energy consumption and toxic accumulation of metabolic byproducts (Kundra et al., 2020). When cells enter the stationary growth phase in stressful conditions, the stringent response (SR), regulated by ppGpp response RelA and SpoT homologous enzymes (Schäfer et al., 2020), is activated for the remodeling of gene expression, leading to increased survival of bacteria (Kundra et al., 2020).

In the next experiments, we followed the growth and the expression of *yfp* (pAG032) and *rfp* (pBSibp) that are under the control of P*rpsJ* and P*ibpfxs* promoters, respectively in the strains grown in LB or minimal medium supplemented with C1 compounds (formate and methanol) or antibiotic, ciprofloxacin. The ciprofloxacin was selected as a positive control for these experiments due to its known cytotoxic impact on bacterial cells via disrupting the cell membrane structure and binding to DNA gyrase and topoisomerase IV and, consequently, inhibiting replication and transcription (Chen et al., 1996). Notably, in the presence of ciprofloxacin, RecA protein activates the SOS response additionally to ppGpp synthesis and SR in order to repair caused DNA damage (Sassanfar and Roberts, 1990; Friedberg et al., 1995).

3.2.1 Reporter plasmid pAG032

The plasmid pAG032 has the *yfp* gene under the control of P*rpsJ* promoter, responsible for the detection of stringent response. It was hypothesized that the expression level of *yfp* depends on the growth rate and is negatively affected by the increasing levels of ppGpp.

The experiments were performed with wt and pAG032 carrying strains in LB medium (Fig. 12) or minimal (Fig. 13) medium. Methanol and formate were added to follow the effect of C1 compounds on KT2440 and engineered strains CSA and CSA_pMnec. Measurements were performed in microtiter plates and A_{580} and YFP were registered every half an hour for 24 hours to obtain growth curves and stress response levels.

The growth of wt strains in LB media revealed that the CSA has the lowest yield in all used media (Fig. 12, 13) and differently from other strains KT2440 was not able to grow in ciprofloxacin supplemented LB (Fig. 12A). The same pattern was observed with a plasmid carrying strains (Fig. 12D, 12E, 12F) indicating that bearing the plasmid is not causing the strains additional metabolic load. Although the strains CSA and CSA_pM were specifically modified by Bruinsma et al. 2023, by the introduction of the reductive glycine pathway (rGlyP) to increase the rate of formate degradation, the growth yield in C1 compounds supplemented media was not higher than in LB. Why do CSA and CSA_pAG032 have the lowest biomass yield among the tested strains? As the experiments were performed in microtiter plates one possible explanation can be a lack of oxygen as rGlyP is the aerobic pathway. Unlike KT2440 the C2 module of rGlyP was deleted from CSA and that may cause the diminish of growth. In the case of CSA_pMnec and CSA_pMnec_pAG032, the growth yields are close to KT2440 and KT2440_pAG032 (Fig. 12A and 12D), it may be that the overexpression of C1 module genes from the introduced pMnec plasmid somehow restored the growth. Although methanol dehydrogenase and formaldehyde dehydrogenase encoding genes were also introduced into the pM plasmid the biomass yield of CSA_pMnec did not increase in methanol-supplemented LB medium (Fig. 12C, 12F).

Additionally, both CSA and CSA_pMnec (wt and pAG032 transformed) seem to obtain higher tolerance of even resistance to ciprofloxacin, as they started growing almost at the same time as in other used media, and expression of YFP under control of P*rpsJ* is significantly lower compared to KT2440_pAG032. This might be because of spontaneous mutations in genes encoding regulators of efflux pumps that pump efficiently antibiotics out

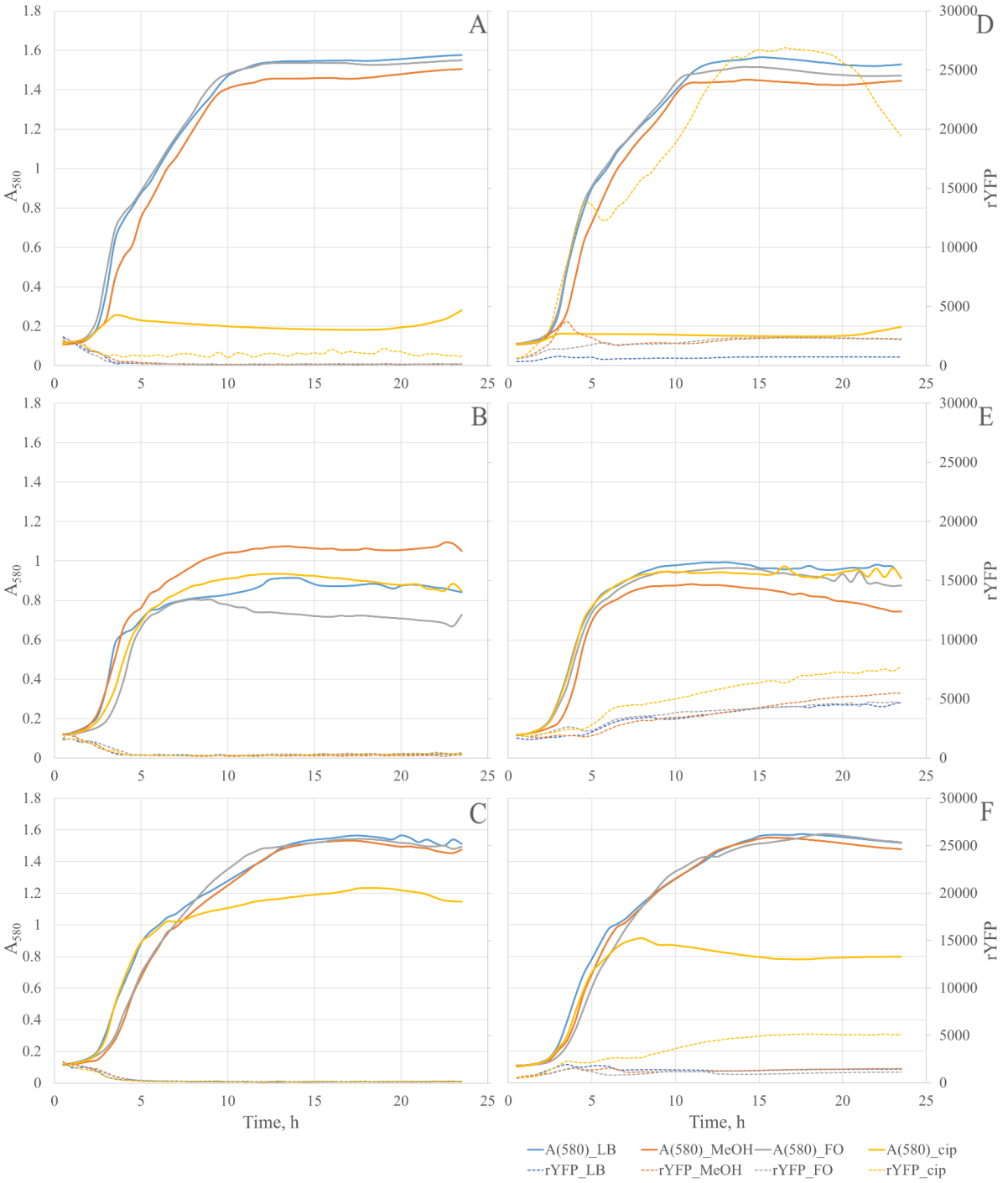

Figure 12. The growth (A_{580}) of strains KT2440 (A), CSA (B), CSA_pMnec (C), and transformed with pAG032: KT2440 (D), CSA (E), CSA_pMnec (F), in LB and LB supplemented with methanol (MeOH; 5%), formate (FO; 30 mM) or ciprofloxacin (CIP; 0.04 µg/ml), and relative fluorescence (rYFP) of cells (505/538 nm). The averages of technical parallels (7) of one biological experiment are shown (no deviations presented to avoid noise).

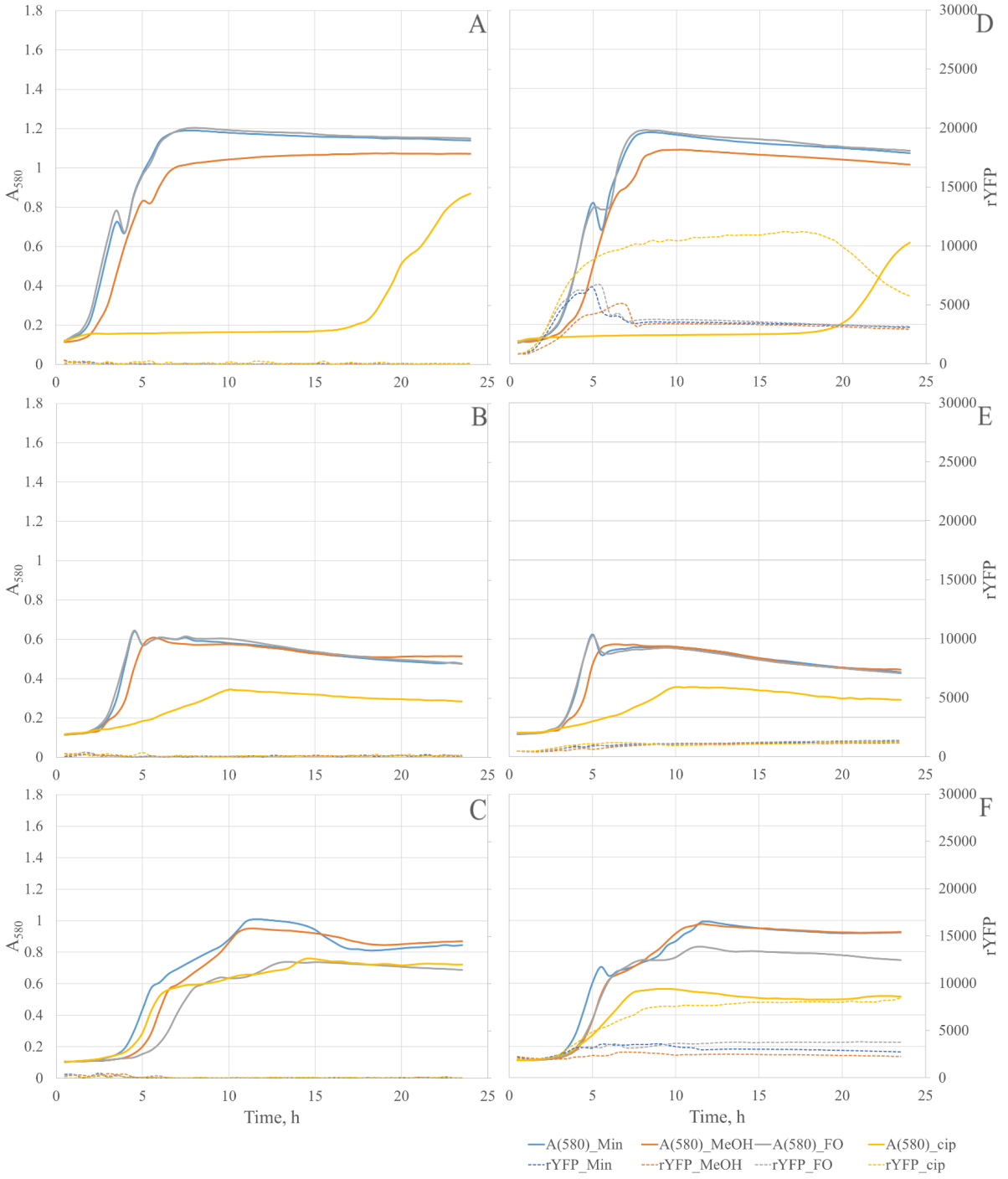

Figure 13. The growth (A_{580}) of strains KT2440 (A), CSA (B), CSA_pMnec (C), and transformed with pAG032: KT2440 (D), CSA (E), CSA_pMnec (F), in minimal and minimal supplemented with methanol (MeOH; 5%), formate (FO; 30 mM) or ciprofloxacin (CIP; 0.04 µg/ml), and relative fluorescence (rYFP) of cells (505/538 nm). The averages of technical parallels (7) of one biological experiment are shown (no deviations presented to avoid noise).

from the cells or during the deletion of C2 module somehow the outer membrane proteins or cell wall have been affected. In *E. coli* it has been shown that due to mutagenic changes and the allele recombination between chromosomes, as the outcomes of the SOS response, the ciprofloxacin-resistant strain eventually forms (Bos et al., 2014). Ponmalar et al., 2022 showed that *E. coli*'s cell membrane dynamics and morphology changed during the contact with ciprofloxacin possibly due to disrupted lipid packing or altered lipid composition.

The inhibition of growth caused by ciprofloxacin in KT2440 resulted in an increase of the rYFP (Fig. 12D) that reached the maximum level after 13 h. The rYFP started to decrease approximately at 21 h and at the same time cells started to grow and A_{580} increased. This type of antibiotic inhibits DNA replication, influences membranes by increasing membrane fluidity, and induces SOS response (Qin et al., 2015; Ponmalar et al., 2022). Gawin et al., 2019 showed that the expression of YFP that is under the control of *PrpsJ* increases when cells grow slower and that is in accordance with our results. Under nutrient depletion or other stress conditions, the concentration of ppGpp increases, and DNA replication and protein synthesis are reduced, shifting cells toward reduced growth and stress survival. Burgos et al., 2017 showed that ppGpp is a negative regulator of rRNA promoters, but the concentration of ppGpp is in the stationary growth phase too low to effectively regulate the transcription of rRNA, and during nutritional shifts the inhibition of P*rpsJ* by ppGpp is indirect. Stress from heat shock or antibiotics causes ppGpp to rise up quickly to the threshold required to mount the SR that changes cell physiology from a growth mode to a survival mode (Kundra et al., 2020; Dalebroux et al., 2010).

The results demonstrated in Figure 13 show that compared to the LB medium (Fig. 12) the biomass yield of bacterial strains grown in a minimal medium was lower, despite the fact that the minimal medium was supplemented with serine to support the growth of genetically modified auxotrophic strains CSA and CSA_pMnec. Correspondingly, the rYFP values were lower than in LB (approximately 2.5-fold). With CSA the rYFP increased until the cells reached the stationary phase, but there was no difference between the applied media (Fig 13E). At the same time, ciprofloxacin induced the expression of the *yfp* in the strains CSA_pMnec and KT2440 (Fig 13D, 13F). In a methanol-supplemented minimal medium, the growth yield was lower and the lag phase was longer than in a minimal medium in the case of the KT2440 and KT2440_pAG032. The delayed growth was also detected with CSA and CSA_pMnec (both with and without the pAG032) when methanol and formate were added to the medium. At the same time only with CSA_pMnec strains the growth yield decrease was observed in formate-minimal medium. From the growth curves can be seen that growth in a

glucose-containing medium does not occur smoothly as there is the second logarithmic phase with a lower growth rate (strains KT2440 and CSA_pMnec). In the case of the strain CSA, there is only one logarithmic phase, and the maximum yield is similar to the plateau of diauxic growth possessing strains.

To sum up, the expression of *yfp* that is controlled by P*rpsJ* in the plasmid pAG032 is induced in studied strains grown in LB or minimal medium supplemented with C1 compounds or antibiotic. The strongest effect was obtained with ciprofloxacin-containing media where rYFP values were highest due to the hindered growth of the KT2440 and CSA_pMnec. Is it caused by ppGpp or stringent or SOS responses needs more investigation, but it is certain that methanol and formate do not cause at the used concentrations so big stress on strains.

A previous study conducted by Gawin aet al., 2019 proposed the hypothesis that the σ^{32}-dependent P*ibpfxs* promoter can be suitable to measure chemical-induced stress as well, as it reacts, for instance, to the presence of amino acid analogues, which interfere with protein synthesis, enzymatic activity and disrupt metabolic pathways, causing protein misfolding, as other chemicals usually do. For that purpose, the pBSibp plasmid (gift of Riho Teras) with *rfp* expression controlled by P*ibpfxs*, responding to heat shock and potentially protein misfolding from chemicals will be used next.

3.2.2 Reporter plasmid pBSibp

The pBSibp plasmid (Table 2) with encoded *rfp* expression controlled by σ^{32}-dependent P*ibpfxs* tandem promoter was hypothesized to be suitable for the measuring of chemical-induced stress because of promoter capability of reacting to misfolded proteins and membrane stress (Gawin et al., 2019).

In general, growth and stress response levels measured in relative red fluorescence (rRFP) units of wild-type bacterial strains in LB did not exhibit any significant difference between the strains (Fig. 14A-C, 15A-C). The NC strain (KT2440 transformed with pBSibp, but not able to express RFP) shared analogous behaviour with the wild-type strain KT2440 (Fig 14A, 15A) and exhibited similar growth and stress response patterns in both LB (Fig. S1A) and minimal medium (Fig. S1B) conditions. However, the longer lag phases indicate that bearing the plasmid has a metabolic burden on cells. Compared to data from the previous experiment (3.2.1) with pAG032 transformed cells where the rYFP increases (Fig. 12D-F, 13D-F), but in the pBSibp transformed cells rRFP decreases (Fig. 14D-F, 15D-F) approximately to the end of the logarithmic growth phase. In strains KT2440_pBSibp and CSA_pMnec_pBSibp the rRFP

level in C1 compound-supplemented media did not increase significantly and was at the same level as in LB medium-grown cells (Fig 14D, 14F). While the rRFP level in CSA_pBSibp increased in the stationary growth phase in all tested media (Fig. 14E, 15E). Supplementation of the medium with ciprofloxacin was stressful for the KT2440_pBSibp as the cells were not growing and the rRFP values were high throughout the experiment (Fig 14D, 15D). At the same time, CSA and CSA_pMnec started to grow in this medium at the same time as in LB (although the biomass yield was lower) and rRFP increased in the stationary phase (Fig 14E, 14F) but was almost at the same level as in KT2440_pBSibp (Fig 14D).

Experiments performed in a minimal medium with pBSibp-transformed cells resulted in lowered biomass yield values compared to LB-grown cells. Moreover, taking a closer look at the behaviour of CSA and CSA_pMnec (both wild types (Fig. 15B, 15C) and transformed with pBSibp (Fig. 15E, 15F)), it becomes evident that lag phase length has significantly increased, which is a sign that the growth of these bacteria in the minimal medium was inhibited when they harboured pBSibp. The most significant effect was observed in two plasmids, pMnec and pBSibp, carrying strain CSA_pMnec where the lag phase length increases in C1 compound containing media was remarkable. In these experiments it is clearly seen that the start of the decrease of rRFP correlates with the length of the lag phase. So, when cells have overcome the stress caused by chemicals the P*ibpfxs* controlled expression is repressed and rRFP decreases.

In general, results suggest that the introduction of the plasmid pBSibp (Fig. 14, 15) has supported the theory, that, despite the *rfp* expression is controlled by σ^{32}-dependent P*ibpfxs* promoter, it is able to respond not only to heat-induced but to protein misfolding from chemical-induced stress as well. However, the pBSibp plasmid is not suitable for ppGpp-mediated stress response, because it does not have a promoter that would be sensitive to ppGpp. However, it is suitable for the detection of non-heat shock stress sensed by RpoH or RpoE as a side effect of misfolded proteins or membrane disturbance by chemical factors. The transformed pBSibp plasmid showed the increased load on bacterial adaptation mechanisms by increasing the duration of the lag phase, not very noticeably in the LB medium, but rather significantly in the minimal medium.

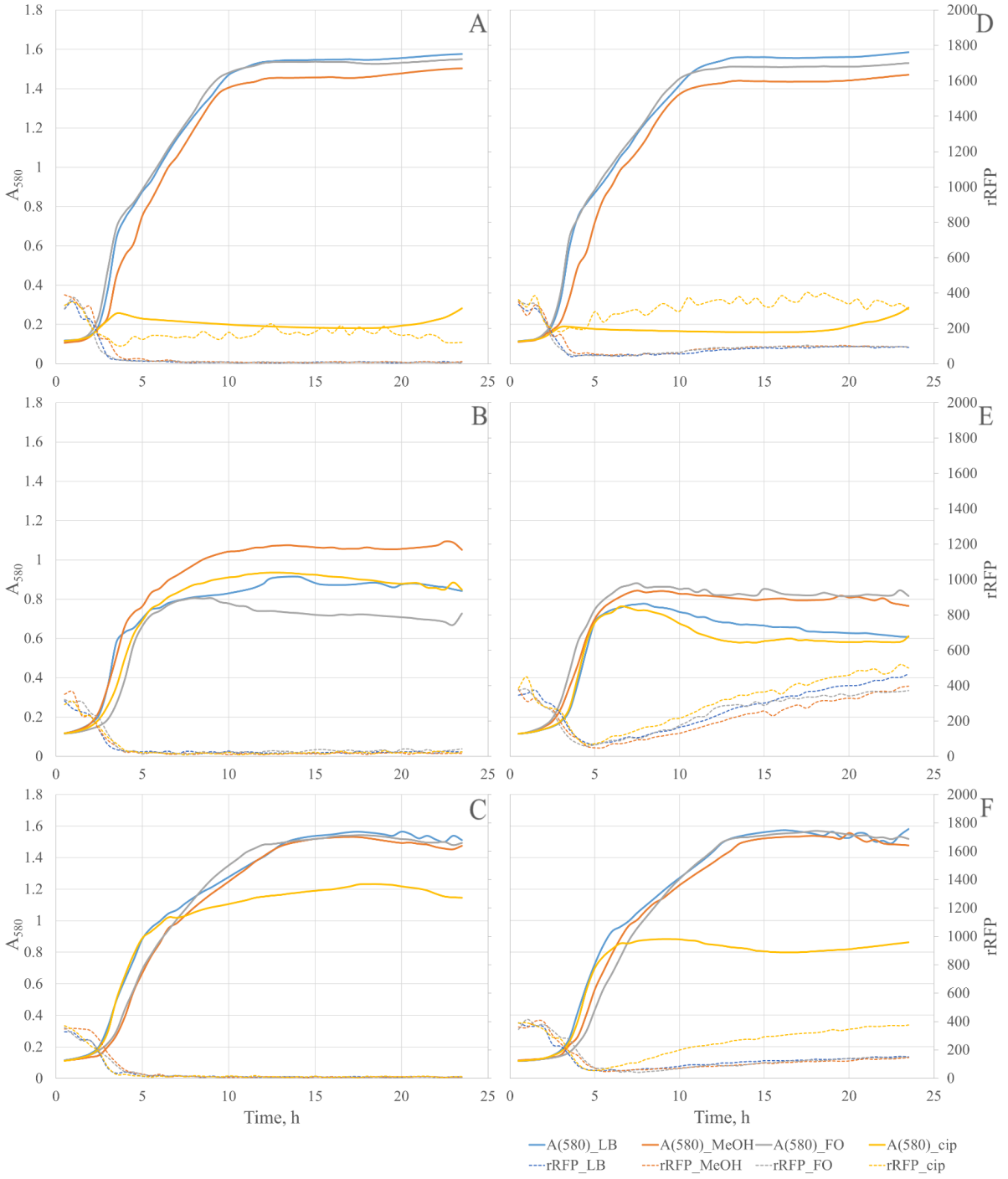

Figure 14. The growth (A_{580}) of strains KT2440 (A), CSA (B), CSA_pMnec (C), and transformed with pBSibp: KT2440 (D), CSA (E), CSA_pMnec (F), in LB and LB supplemented with methanol (MeOH; 5%), formate (FO; 30 mM) or ciprofloxacin (CIP; 0.04 µg/ml), and relative fluorescence (rRFP) of cells (570/600 nm). The averages of technical parallels (7) of one biological experiment are shown (no deviations presented to avoid noise).

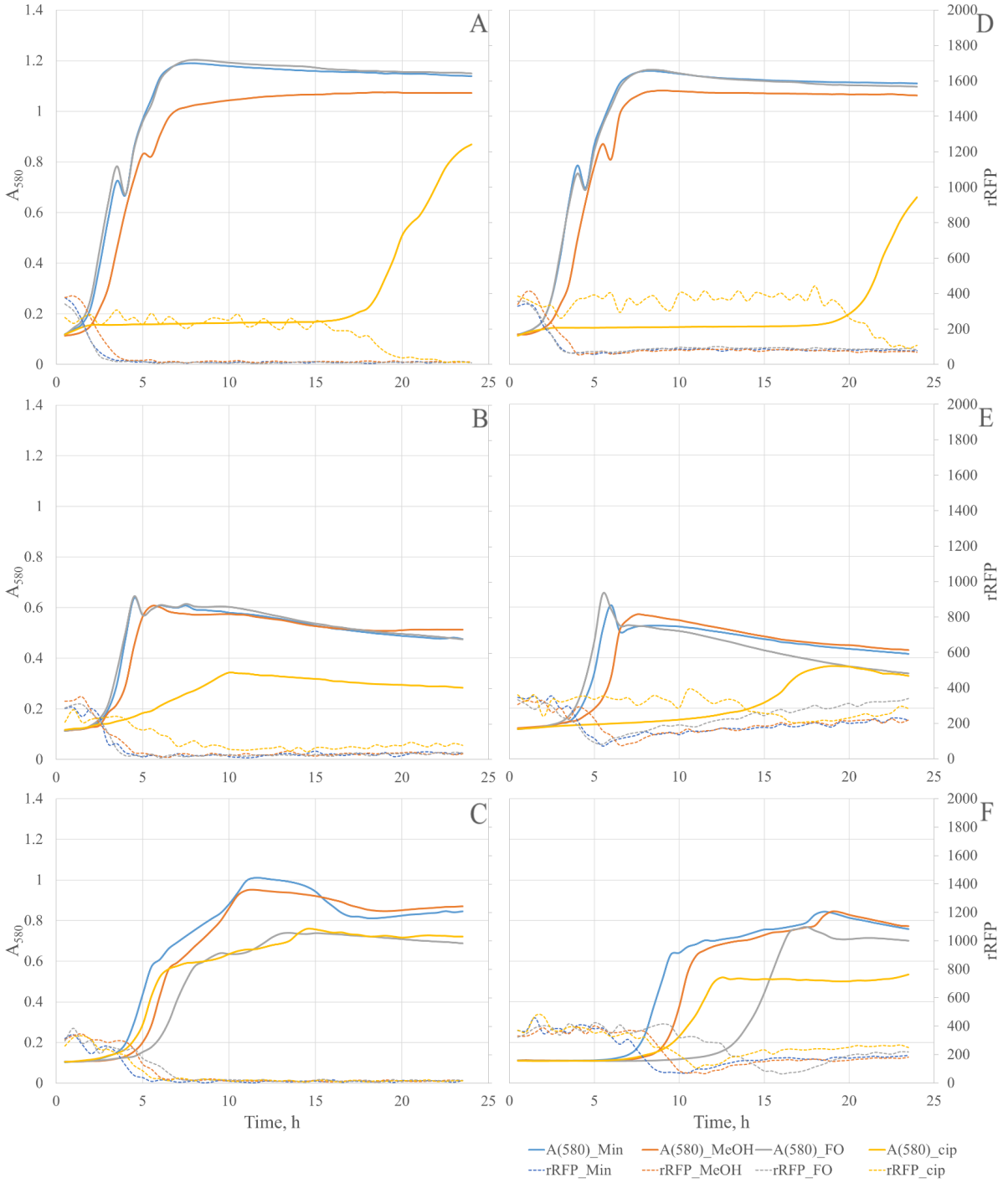

Figure 15. The growth (A_{580}) of strains KT2440 (A), CSA (B), CSA_pMnec (C), and transformed with pBSibp: KT2440 (D), CSA (E), CSA_pMnec (F), in minimal and minimal supplemented with methanol (MeOH; 5%), formate (FO; 30 mM) or ciprofloxacin (CIP; 0.04 µg/ml), and relative fluorescence (rRFP) of cells (570/600 nm). The averages of technical parallels (7) of one biological experiment are shown (no deviations presented to avoid noise).

SUMMARY

Pseudomonas putida strains C1-S-Aux (CSA) and C1-S-Aux_pMnecator (CSA_pMnec) were specifically engineered by Bruinsma et al., 2023 for assimilation of C1 compounds: methanol and formate, to gain the capability of using substrates as a renewable feedstock.

To test the productivity and durability of these strains for future usage in industrial settings, taking into account possible accidents, the stress response of wild-type *Pseudomonas putida* KT2440 was compared to C1 compounds utilizing strains CSA and CSA_pMnec. Additionally, the different response reporter systems were tested, and, finally, the growth and relative fluorescence dynamics in two C1 supplemented media: LB and minimal medium, were analysed.

During the experiments following observations and conclusions were made:

1. Plasmid-based reporter system pAG032 is not suitable for measuring the genuine heat shock response in *P. putida* strains, as naturally produced fluorescent bacterial proteins and CFP's fluorescence spectra overlap.
2. pBSibp is suitable for measuring heat shock response in *P. putida*, as it gives much higher ratios in response to temperature elevation than pAG032.
3. Plasmid pBSibp has probably a metabolic burden on cells.
4. From C1 chemicals methanol inhibited the growth of bacterial cultures more than formate. On methanol and formate-supplemented media, the strains CSA and CSA_pMnec did not give an increase in biomass compared to LB or minimal medium.
5. P*rpsJ* is suitable for measuring ppGpp-mediated stress response, as this is a ppGpp-regulated ribosomal protein promoter, which reacts to stringent response as a result of ppGpp activation.
6. P*ibpfxs* promoter introduced into pBSibp is suitable for measuring chemical-induced stress response, as it reacts to protein misfolding and membrane damage, which are the consequences of chemical damage.

To summarize, the studied strains CSA and CSA_pMnec, can tolerate rather high concentrations of methanol and formate, however, the yield produced by these strains during the experiments does not significantly outperform the wild-type's *Pseudomonas putida* KT2440. Although both tested reporter systems worked, a promoter must be found that reacts only to one stress to obtain more precise stress response data.

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SUPPLEMENTARY MATERIAL

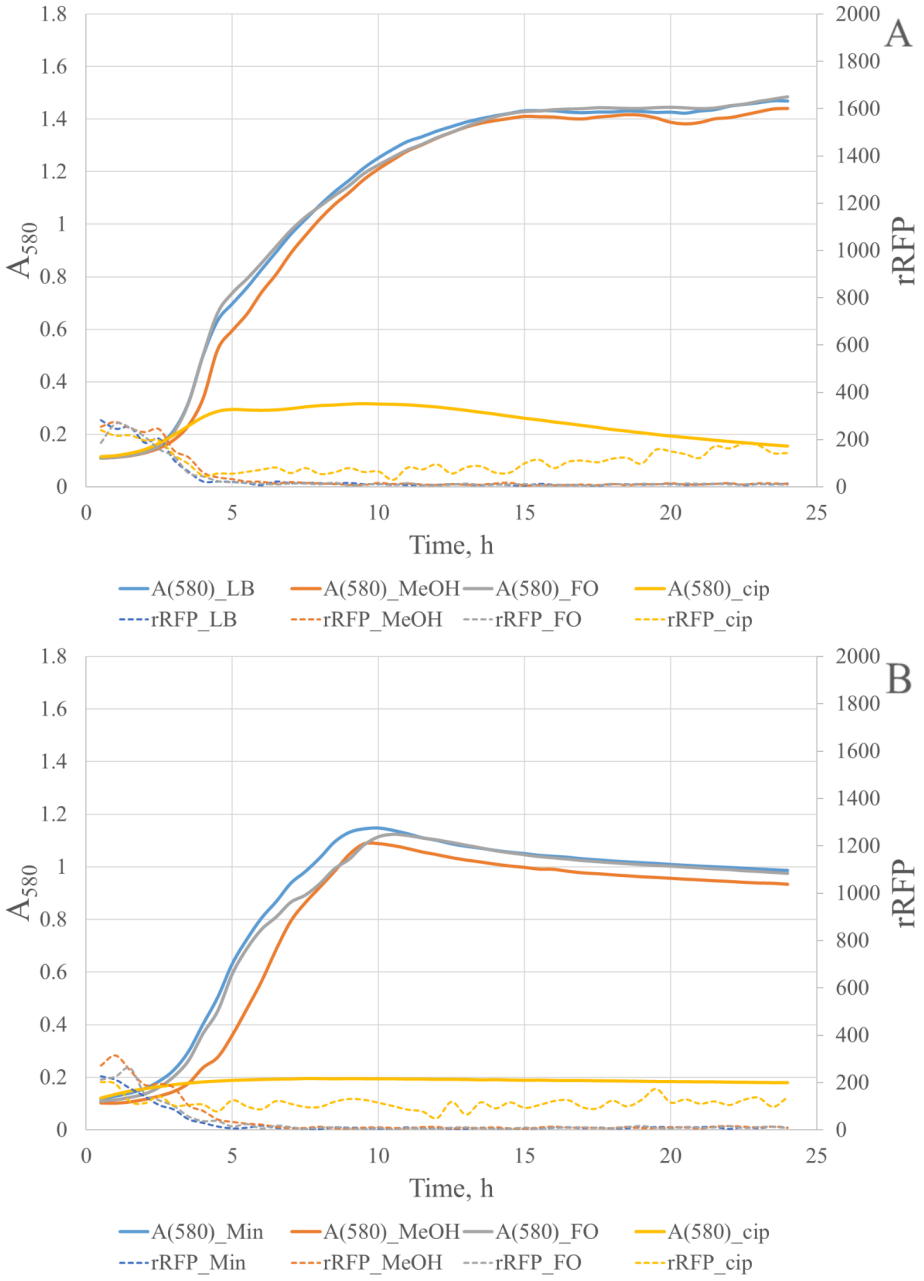

Figure S1. The growth (A_{580}) of strain KT2440 transformed with pBSibp possessing ampicillin resistance but not able to produce RFP (NC) in LB (A) and minimal (B) medium, both supplemented with methanol (MeOH; 5%), formate (FO; 30 mM) or ciprofloxacin (CIP; 0.04 µg/ml), and relative fluorescence (rRFP; 570/600 nm) of cells. The averages of technical parallels (7) of one biological experiment are shown (no deviations presented to avoid noise).

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