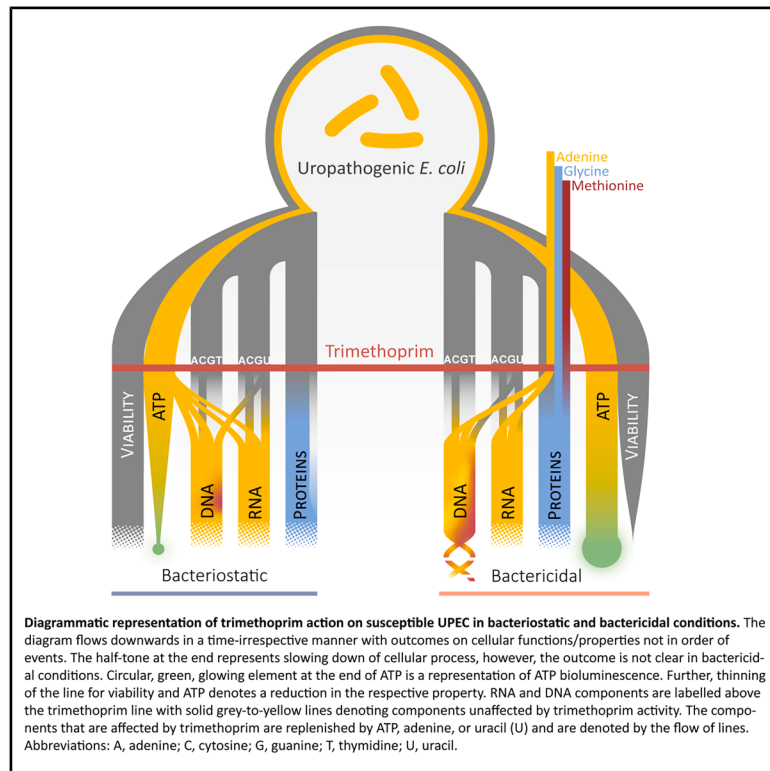


Metabolic reconstruction reveals ATP salvage as a key response to trimethoprim treatment

Graphical abstract



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In brief

Biochemistry; Microbiology; Metabolomics

Highlights

- Metabolic reconstruction shows trimethoprim activity depends on metabolite availability
- Bacteria salvage ATP to restore the purine pool and repair DNA during trimethoprim stress
- Media composition shapes trimethoprim efficacy with implications for personalized medicine



Article

Metabolic reconstruction reveals ATP salvage as a key response to trimethoprim treatment

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SUMMARY

Trimethoprim is a clinically important antibiotic for treating urinary tract infections, yet its mechanism of killing remains elusive due to a cascade of affected metabolic reactions. Metabolites available in growth media can selectively counteract trimethoprim, which affects the interpretation of results. We sought to understand the full scope of trimethoprim's impact on *Escherichia coli* metabolism and how metabolite availability affects trimethoprim outcomes. We applied flux balance analysis on a genome-scale metabolic model of *E. coli* to simulate trimethoprim activity under bacteriostatic and bactericidal conditions. Our results suggested that in the absence of environmental purines or nucleosides, trimethoprim induces salvage of ATP to repair DNA. We experimentally validated the result with ATP bioluminescence screening of 96 clinical *E. coli* isolates. Therefore, the choice of growth media composition significantly changes the outcome of the trimethoprim challenge and opens opportunities for personalized medicine.

INTRODUCTION

Urinary tract infections (UTIs) place a substantial burden on health care facilities and the quality of life for patients experiencing severe pain and discomfort.¹ One antibiotic to treat UTIs, trimethoprim, is of clinical significance as the most cost-effective treatment option and is still used routinely despite rising resistance rates.^{2,3} Trimethoprim targets an important group of carbon-shuttling compounds called folates that are essential for providing carbon units for the synthesis of three of the four components of DNA (adenosine, guanosine, and thymidine), two of the four components of RNA, and methionine (Figure 1).⁴ Folates also provide the formyl group for protein synthesis initiatory *N*-formylmethionine (tRNA^{Met}). Therefore, trimethoprim directly affects DNA, RNA, and protein synthesis by limiting the availability of carbon-carrying folates.

As these components of DNA, RNA, and proteins are also precursors for other metabolites, it is challenging to draw strong conclusions on the full extent of trimethoprim's activity.⁵ This is further complicated by trimethoprim having bacteriostatic or bactericidal effects depending on the metabolites available in growth media.⁶ Bacteriostatic trimethoprim activity depends on purines or nucleotides being excluded from growth media for trimethoprim to visibly slow or arrest cellular growth.^{7,8} Conversely, bactericidal activity requires the presence of a purine or nucleoside, and the amino acids glycine and methionine. The killing mechanism is suspected to be caused by the inhibition of DNA synthesis via thymidine starvation, while protein and RNA synthesis are alleviated. Faltered DNA synthesis and

repair triggers programmed cell death via the suicide module *mazEF* and is analogous to thymineless death.^{5,6,9,10} The role of glycine is unknown, with some suggestions that it plays a regulatory role in the bactericidal action, but it lacks sufficient evidence to rule out other amino acids that can replace glycine.^{5,6} Methionine replenishes trimethoprim-induced methionine depletion, thereby enabling protein synthesis, which is essential for bactericidal activity.⁶ Therefore, a bactericidal outcome appears to require DNA, RNA, and protein synthesis via the availability of purine, glycine, and methionine.^{6,7} Thymidine also plays an important role as a putative trimethoprim inhibitor; most organisms have a thymidine kinase to synthesize deoxythymidine monophosphate (dTMP) from thymidine, which limits trimethoprim's inhibition on DNA synthesis.¹¹ The counteractive property of thymidine is only evident in bactericidal conditions yet metabolomics and protein expression analysis in bactericidal conditions suggest thymidine can only partially counteract trimethoprim.^{5,6,12–15}

As the full scope of trimethoprim's activity on bacterial metabolism is unknown, we sought to better understand trimethoprim's mode of action through genome-scale metabolic (GSM) modeling. The challenge with investigating folates and the trio adenine, glycine, and methionine is that they are highly connected metabolites. For example, adenine is involved with 20 reactions (excluding transporters), glycine with 49, and L-methionine with 56 (according to EcoCyc v27.1 *E. coli* K-12 substr. MG1655 database when accessed on 11/08/2023). Nonetheless, this challenge can be approached *in silico* with GSM modeling, which provides a tractable representation of



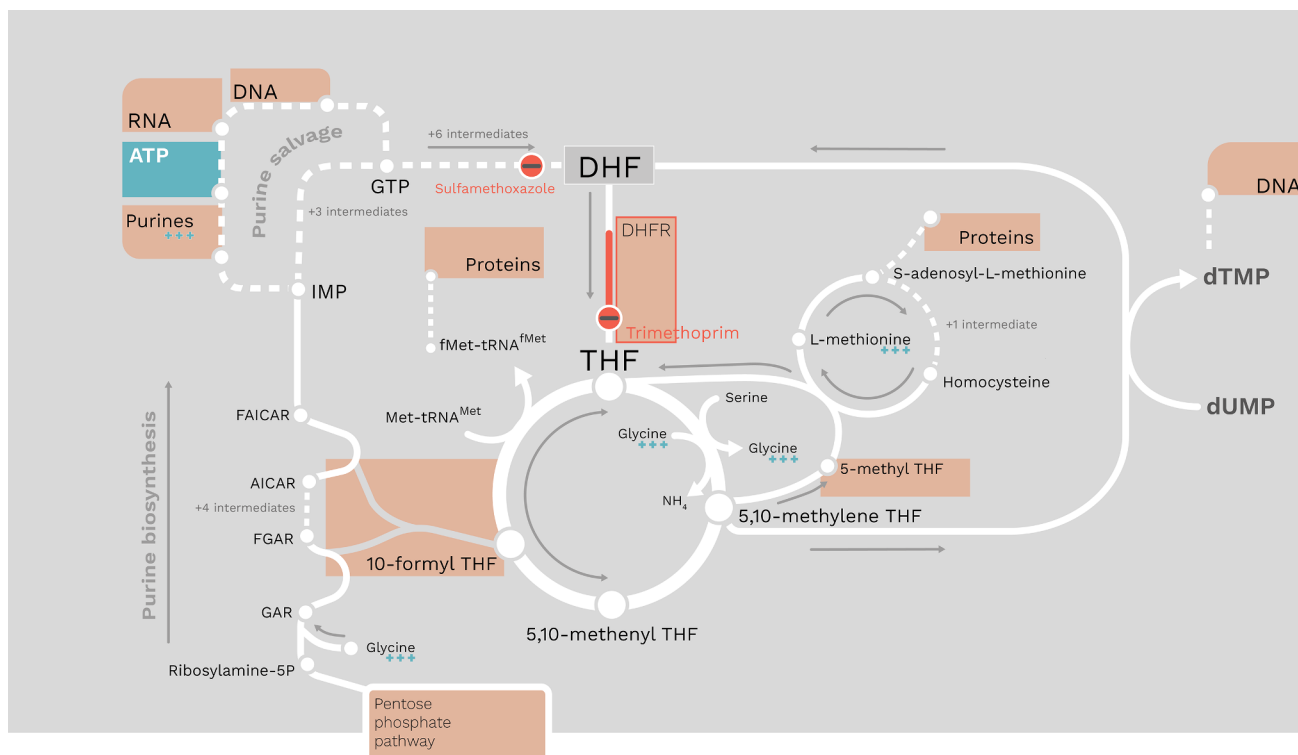


Figure 1. A simplified network map of the folate pool and relevant pathways, including purine biosynthesis and salvage, and folate biosynthesis

White lines denote reactions and arrows for direction; sets of reactions have adjacent dark-grey arrows for direction. Dashed white lines denote several reactions and are condensed for clarity, with labels stating the number of intermediary reactions. Red circles with a black bar denote reactions where trimethoprim and sulfamethoxazole exert their anti-folate effects, and metabolites/macromolecules/reactions with a light brown box denote components of metabolism affected by anti-folate drugs. Metabolites that are present in growth media, which elicit a bactericidal activity, are annotated with three teal plus signs. ATP is highlighted with the same teal color to emphasize its position in the network. Dihydrofolate reductase (DHFR) is the only reaction labeled in the network map due to its significance as the target for trimethoprim. Reactions and pathways are derived from EcoCyc v21.

an organism's metabolic network. Here, we focus on uropathogenic *Escherichia coli* (UPEC), the predominant etiological agent of reported UTIs internationally, causing 77% of UTIs.¹⁶

RESULTS

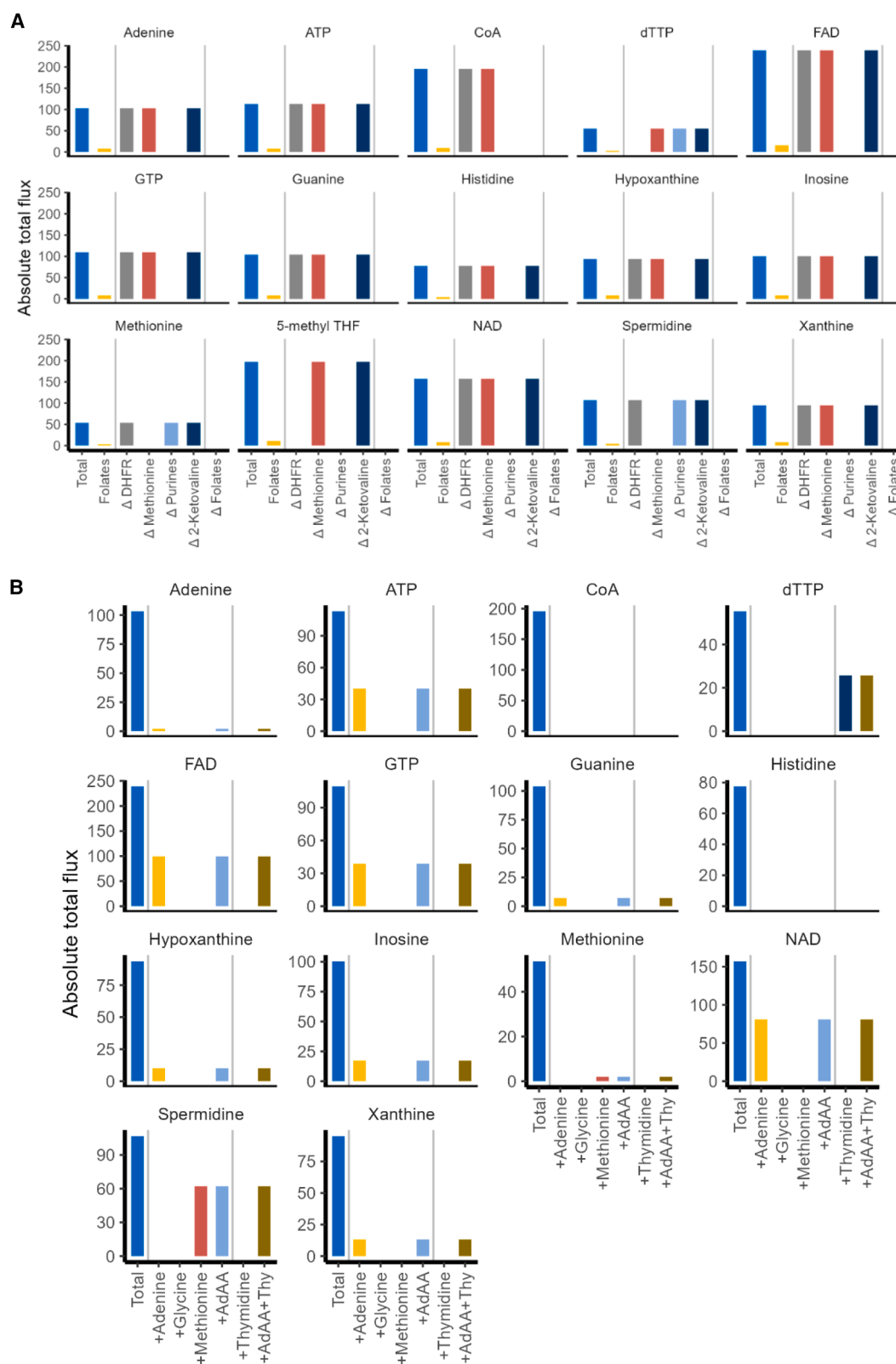
Trimethoprim disruption of biomass synthesis in bacteriostatic conditions

Trimethoprim is known to inhibit the synthesis of some amino acids and purines; therefore, it was prudent to replicate this *in silico* with a GSM model (Equation 1). By mimicking trimethoprim's constraints on folate reactions *in silico*, we found trimethoprim directly affected the synthesis of 15 biomass components (excluding derivatives such as NAD:NADH): 8 purine and (deoxy)nucleotide triphosphates, 2 amino acids, 3 cofactors, one amine compound, and folates (Figure 2A).

This analysis highlighted some acute differences between what would be expected *in vivo* and how to reproduce it *in silico*. Most notably, preventing flux via DHFR (the target enzyme for trimethoprim) only affected the synthesis of folates (in the form of 5-methyl THF) and dTTP. It was then suspected that some biomass components utilize folates but do not require DHFR for their synthesis under steady state. Indeed, the other biomass

components that required folates were dependent on the folate “pool” rather than DHFR activity. This implied that the folate pool had a conserved moiety (a group of compounds that are cyclically interconverted to one another) as THF could acquire a carbon unit from serine or glycine, donate that to the synthesis of a metabolite, and revert to THF. This also made investigating trimethoprim a challenge as FBA does not allow for the depletion or accumulation of internal metabolites under steady state. A second concern was that folates could not be salvaged in the model. Therefore, when synthesizing folates *de novo*, the only means of balancing the solution was by exporting folates as 5-methyl-THF, which is one of the biomass components (Table S1).

Nonetheless, the biomass components that directly required folates for their synthesis were identified. Of particular interest was ATP, which required folates for *de novo* synthesis via enzymes involved in the purine biosynthesis pathway that utilized 10-formyl THF. The dependence on folate in histidine and spermidine synthesis was unexpected as this is not well reported in the literature. However, histidine was derived from parts of the purine biosynthesis pathway that involved folates (see review by Winkler and Ramos-Montañez¹⁷). Upon closer inspection, the biosynthesis of spermidine relied on S-adenosylmethionine



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(SAM), which required folates for its biosynthesis via the synthesis of L-methionine.¹⁸ All purines (and their nucleoside [triphosphate] counterparts) and cofactors NAD, FAD, and CoA require folates for their biosynthesis via GAR formyltransferase and AICAR transformylase (Figure 2A).

Supplementation with adenine, glycine, methionine, or thymidine counteracts trimethoprim selectively

To better understand the relevance of adenine, glycine, and methionine in eliciting bactericidal trimethoprim activity and how thymidine counteracts trimethoprim, their capacity to counteract trimethoprim-induced constraint on biomass synthesis was investigated (Equation 2). Under the condition of zero flux via folate-associated reactions (Table S5), adenine supplementation permitted the synthesis of all purines and nucleosides (Figure 2B). Cofactors FAD and NAD were also restored, as GTP was the precursor for FAD, as described in the “flavin biosynthesis I (bacteria and plants)” pathway in EcoCyc v27.1, and NAD required ATP for its synthesis. This suggested that adenine restored the purine, ATP, and cofactor synthesis under trimethoprim challenge. In contrast, the synthesis of CoA was not restored by supplementation, as the constraint applied to dehydropantoate hydroxymethyltransferase (one of the folate-associated reactions) restricted the synthesis of 2-dehydropantoate, which is a precursor for CoA. While histidine and purines share a common pathway for their synthesis, which requires folates, the supplementation of adenine did not restore histidine synthesis. Glycine alone did not restore the synthesis of any biomass components (Figure 2B). Further, the presence of glycine with adenine and methionine did not alter the absolute total flux for synthesizing any biomass components compared to adenine or methionine alone. Thus, glycine’s relevance in the activity of trimethoprim or as a lone supplement was not immediately clear from this analysis. Methionine restored the synthesis of itself and spermidine, for which methionine is a precursor (Figure 2B). No biomass components were exclusively restored by the combination of adenine, glycine, and methionine, which suggests these metabolites selectively counteract trimethoprim. Thymidine allowed the synthesis of dTTP when all folate-associated reactions were void (Figure 2B). Indeed, none of the bactericidal-promoting metabolites altered the total flux required to synthesize dTTP when thymidine was present. Thus, thymidine alone counteracted the limitation applied to thymidylate synthase under trimethoprim action. As the reactions involved in synthesizing folates were constrained to zero, the biomass component 5-methyl THF was excluded from this analysis.

Purine supplementation restores the nucleoside pool during trimethoprim-induced constraints on purine biosynthesis

The capacity for a purine to restore the nucleoside pool under trimethoprim challenge was investigated by increasing nucleoside demand (dGTP export) with constraints applied to purine biosynthesis reactions involving folates (GAR formyltransferase and AICAR transformylase). To simulate trimethoprim’s activity on purine biosynthesis, an upper constraint was applied to these two reactions as a proxy for limited active folate availability (Equation 3). Therefore, the aim was to observe how the model responds when dGTP demand exceeds the constraints applied to purine biosynthesis, i.e., whether the model can still synthesize dGTP or rely on media supplementation. In bacteriostatic conditions without purine availability from the media, the model used purine biosynthesis to synthesize dGTP *de novo* until the upper bounds placed on purine biosynthesis were met (Figure 3). Past those bounds, no feasible solution was available for synthesizing purines. Glycine supplementation alone was not sufficient for surpassing the bounds, but adenine supplementation enabled the model to continue making dGTP through adenine salvage until the limited availability of glucose became restrictive. The same applied to bactericidal conditions; however, the availability of glycine as a carbon source enabled the model to reach double the maximum flux of dGTP than with adenine alone. Also, methionine was not imported in bactericidal conditions, suggesting methionine was not used as a carbon source in these conditions.

Incorporating *in vivo* ATP data suggests ATP is used to restore the nucleoside pool during bacteriostatic trimethoprim action

In vivo absorbance and fluorescence data were integrated into the model to assess how the network utilizes free ATP, measured by bioluminescence and partitioned into growth-related (biomass ATP) and maintenance ATP (mATP) (Figures S4A and S4B). Akin to other biomass components, the biomass ATP was directly proportional to the growth rate. Assuming that mATP could only be salvaged under trimethoprim stress, we incorporated weightings for mATP import in different media compositions to prevent its use during normal growth. Trimethoprim’s activity was simulated by applying weightings to folate-associated reactions, which (unlike directly inhibiting DHFR) allowed for feasible solutions (Equations 8 and 9; Figure S4C). Preliminary observation by Kwon et al.⁵ reported that glycine supplementation into M9 minimal media induces a rapid decline in ATP immediately after trimethoprim exposure

Figure 2. Biomass components affected by the trimethoprim-induced disruption of the folate pool and counteraction by adenine, glycine, methionine, and/or thymidine

(A) Total denotes the absolute total flux through the network to produce 1 unit of metabolite. The folate bar (yellow) indicates the proportion of which involved folate-associated reactions (Table S5). Labels on x axis with Δ denote the total flux through the network when a reaction in the folate pool was restricted to zero flux in the presence of a given metabolite. This includes: dihydrofolate reductase (Δ DHFR); methionine synthase (Δ Methionine); GAR formyltransferase and AICAR transformylase (Δ Purines); dehydropantoate hydroxymethyltransferase (Δ 2-Ketovalline).

(B) Impact of adenine, glycine, methionine, and/or thymidine supplementation on metabolites that require folates for their synthesis when folate-associated reactions are constrained. The total denotes the absolute total flux to synthesize the given metabolite when no constraint was applied to folate-associated reactions and without supplementation. For each metabolite being supplemented, the flux for all folate-associated reactions was set to zero, and shows the absolute total flux of the LP solution. Abbreviations: adenine, glycine, and methionine (AdAA); thymidine (Thy).

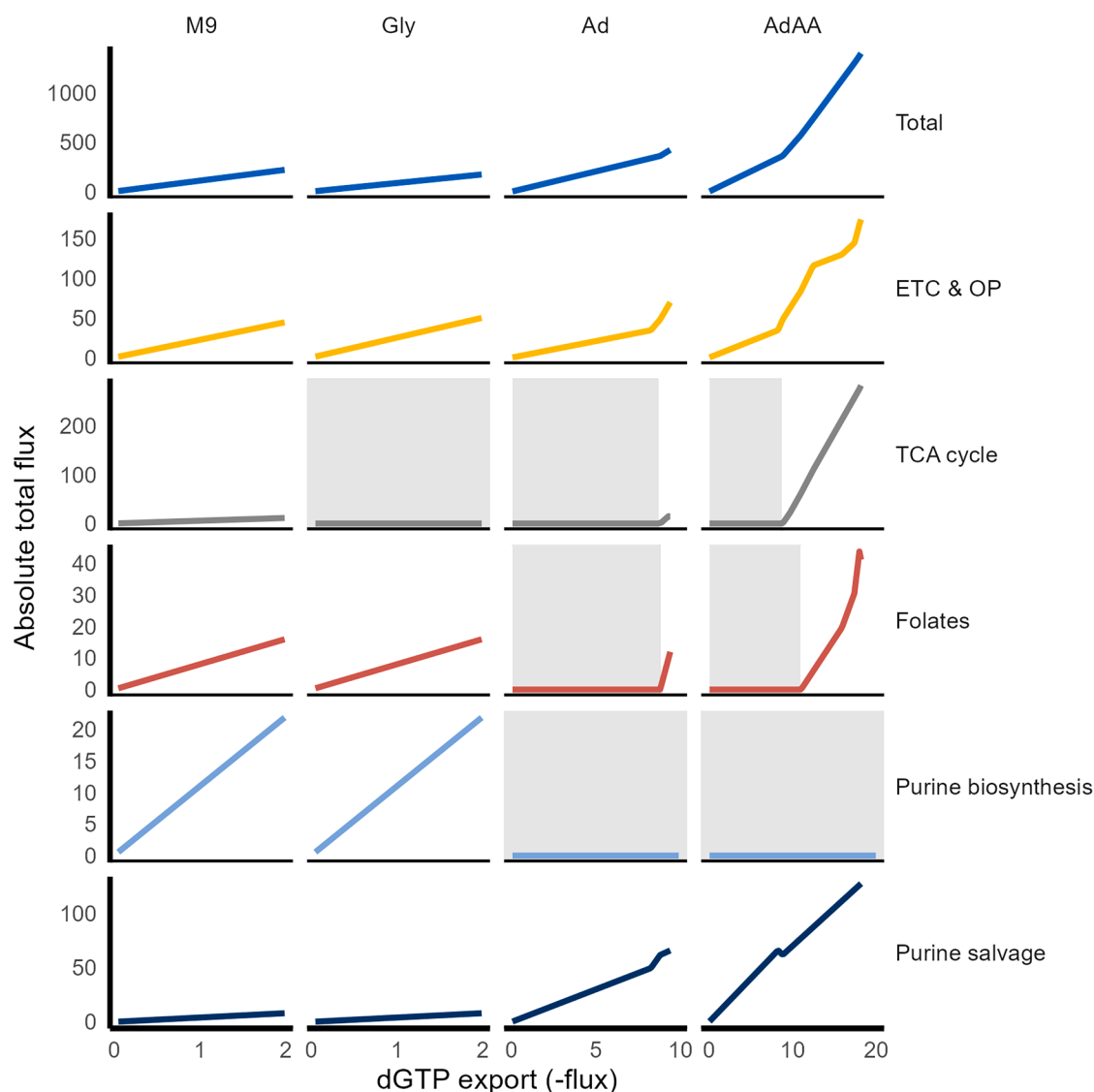


Figure 3. Network response to increasing dGTP demand under trimethoprim-induced *de novo* purine biosynthesis limitation in M9 minimal media with supplementations of glycine (gly), adenine (Ad), or adenine, glycine, and methionine (AdAA)

Each line represents the absolute total flux through a pathway as the flux for dGTP export is increased. Purine salvage under AdAA conditions contains three lines representing the switching of routes as demand increased. Flux via GAR formyltransferase and AICAR transformylase was restricted to a maximum of 2 to imitate trimethoprim-induced purine biosynthesis limitation. Gray backgrounds denote dGTP fluxes where no flux was found for that pathway. Abbreviations: tricarboxylic acid (TCA) cycle; electron transport chain (ETC) and oxidative phosphorylation (OP).

in *E. coli*, and so we included glycine supplementation for this analysis.

We then simulated how the network salvaged mATP under trimethoprim stress in each media condition (Equation 11). In bacteriostatic conditions, mATP was imported after 60 min in trimethoprim-like conditions (Figure 4A). The simulated decline in total external ATP for treated groups compared to control at 75 min mirrored the proportion of ATP decline observed *in vivo* (−32.9% in M9 and −35.9% in M9+Gly; Figure 4B), whereas there was no decline in total ATP in bactericidal conditions. Unlike M9 conditions, where the treated group observed a single large shift in the proportion of mATP (at time of trimethoprim constraint)

before a maintained mATP proportion, there was a constant decline in the %mATP in M9+Gly conditions. By 125 min, mATP accounted for 0.24% of total ATP produced in M9+Gly compared to 36.6% in M9 conditions. In bacteriostatic conditions, a significant portion of imported mATP flux could be attributed to the export flux of mATP (36% in M9 and 25.9% in M9+Gly). Of the flux that was not immediately exported as mATP in both bacteriostatic conditions, 45.8% could be attributed to the export flux of biomass ATP and dATP, and 52.9% attributed to the export flux of GTP and dGTP. In bactericidal conditions, 12.4% of adenine import could be attributed to the export of mATP, and the remaining adenine was used to produce

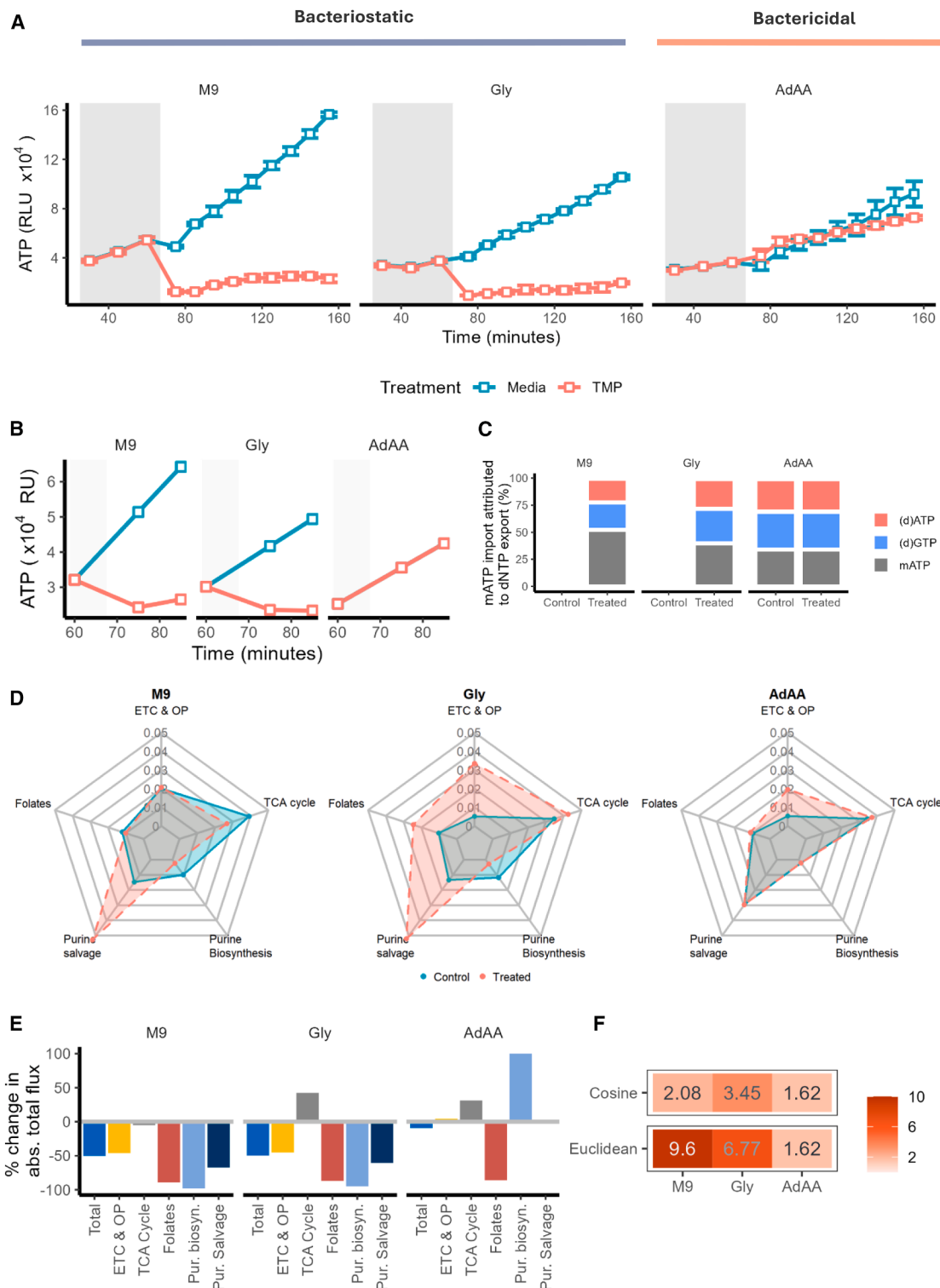


Figure 4. Simulated time-course ATP bioluminescence assay comparing treatment groups in different media conditions after trimethoprim-mimicking stress at the 75-min mark

(A) Time-course ATP bioluminescence assay of a trimethoprim (TMP) susceptible clinical UPEC isolate. Assay was performed in triplicate in M9 minimal media, which was either not supplemented (M9), supplemented with glycine (Gly), or with adenine, glycine, and methionine (AdAA). The gray box denotes baseline

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the same percentages of purines and nucleosides seen in bacteriostatic conditions. This strongly suggested that purines and purine-based nucleosides were the greatest contributor for import of mATP and adenine. Further, purine salvage was a more central component of the network in bacteriostatic media conditions in treated groups compared to the control group, suggesting the ATP salvaged was distributed across the network (Figure 4D).

The switch from *de novo* purine biosynthesis to salvaging mATP resulted in significant network-wide changes in bacteriostatic conditions. M9 media conditions had the smallest change in reaction sets (suggested by cosine distance) compared to the other media compositions, yet had the greatest change in flux within the network (suggested by Euclidean distance) (Figure 4F). Whereas the change in reaction sets and overall flux between control and treated groups in M9+Gly and AdAA conditions were comparable and appear to be attributed to the shift from glycine to glucose and ammonium. When constraints were applied to the folate reactions, this coincided with a decline in the absolute total flux through folate-associated reactions (Table S5) in all media conditions (M9: −91.0%, M9+Gly: −63.6%, M9+AdAA: −68.1%; Figure 4E).

Role of glycine in trimethoprim challenge

The role of glycine in trimethoprim challenge has remained elusive; we sought to understand its function by clustering pathways on the flow of mass through the network, thereby separating reactions into functional groups. With this approach, we identified glycine as the one-carbon unit donor for THF, amino acids, and pyruvate during trimethoprim challenge (Figure 5). In untreated conditions lacking glycine (bacteriostatic), the metabolites pyruvate and ammonium were used to synthesize serine, which acted as the one-carbon unit donor for THF, which also produced glycine via serine hydroxymethyltransferase. In this scenario, glycine was not recycled by the glycine cleavage system to provide a second one-carbon unit to THF. Instead, it was used as a carbon source to make purines, threonine, and other amino acids that were synthesized from glutamate, such as isoleucine (Figure 5). When folate availability was restricted by applying weightings to folate-associated reactions, the solution had reduced glycine usage; glycine was no longer involved in threonine, amino acid, and purine biosynthesis. Any glycine that was needed (such as biomass glycine for proteins) was produced via serine-glyoxylate aminotransferase.

In all scenarios where glycine was available in the media, the solution involved utilizing glycine to synthesize pyruvate following the L-threonine degradation III pathway and super-pathway of methylglyoxal degradation (Figure 5). When

untreated, serine was still produced from pyruvate and ammonium for serine hydroxymethyltransferase to donate one-carbon units to THF. However, the glycine cleavage system was active, and glycine was directly used to synthesize threonine, other amino acids, and pyruvate via glycine C-acetyltransferase and threonine aldolase. This suggests glycine was an effective precursor for these compounds, but too expensive to synthesize *de novo* as a precursor. Trimethoprim-mimicking conditions did little to influence the utilization of glycine; this removed the utilization of the glycine cleavage system and reduced serine hydroxymethyltransferase. All other uses of glycine were preserved. The addition of adenine and methionine (bactericidal) had no effect on the utilization of glycine, except that glycine was not used as a carbon donor for purine biosynthesis due to adenine salvage (Figure 5).

Experimental validation of ATP salvage during trimethoprim challenge

Experimental validation of modeling results was performed by screening ninety-six randomly selected clinical UPEC isolates (equal numbers of trimethoprim susceptible and resistant) to determine whether trimethoprim leads to energy exhaustion in bacteriostatic conditions. In M9 minimal media (bacteriostatic), there was an average of 29.7% reduction in intracellular ATP for trimethoprim susceptible UPEC compared to a 3.4% increase in intracellular ATP for resistant isolates ($p < 0.001$, CI [0.10, 0.07]) (Figures 6A and 6B). This suggests that susceptible populations are salvaging ATP in response to trimethoprim exposure to a degree that can distinguish them from resistant populations in bacteriostatic conditions (ROC-AUC: 0.817) (Figure 6C). There were two optimal cut-off values of −30% (TPR 0.625, TNR 0.937) and −11% (TPR 0.812, TNR 0.75) for distinguishing these populations, the latter of which had 78.1% agreement with the phenotypic results provided by the Clinical Microbiology Laboratory that provided the isolates. Further, of the media compositions tested, M9 (bacteriostatic) provided the most consistency between repeated sampling, with 2σ in susceptible and resistant groups being $\pm 66.3\%$ and $\pm 51.9\%$, respectively (Figure S5). The supplementation of glycine was comparable to base M9 media with a mean decline of 28.6% in intracellular ATP in susceptible isolates compared to a 1.8% increase for resistant isolates ($p < 0.001$, CI [0.12, 0.06]) (Figures 6A and 6B). Likewise, accuracy in distinguishing phenotypic populations was comparable (ROC-AUC 0.81) at its optimal cut-off of −15% (TPR 0.79, TNR 0.87). In contrast, the presence of adenine, glycine, and methionine, which elicited a bactericidal outcome, did not lead to significant ATP decline in both phenotypic populations (ROC-AUC 0.36). There was a

measurements before the treatment of blank media or trimethoprim was added. Each data point is the mean of 4 biological replicates with error bars show the standard error mean of 95% confidence intervals.

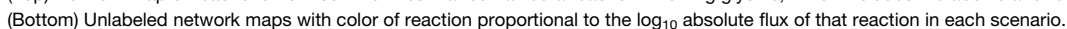
(B) Simulated change in total free ATP (relative units; RU) over time. Gray box indicates baseline measurements with weighting applied to folate-associated reactions at 75 min for treated groups (TMP; trimethoprim).

(C) Proportion of mATP import flux attributed to the export of a given nucleotide.

(D) Centrality (PageRank) of relevant pathways. Values given at 75 min per media group on a radar chart, where each ring is 0.01 steps and colored by treatment group. Solid blue lines and shading denote the control group, and the dashed red line with shading denotes the trimethoprim-treated group.

(E) Percentage change in absolute total flux between treated and control groups at 75 min in relevant pathways.

(F) Cosine and Euclidean distance between flux vectors from LP solutions at 75 min between control and treated groups. Cosine distance was multiplied by 100, and Euclidean distance was divided by 10,000 for clarity.



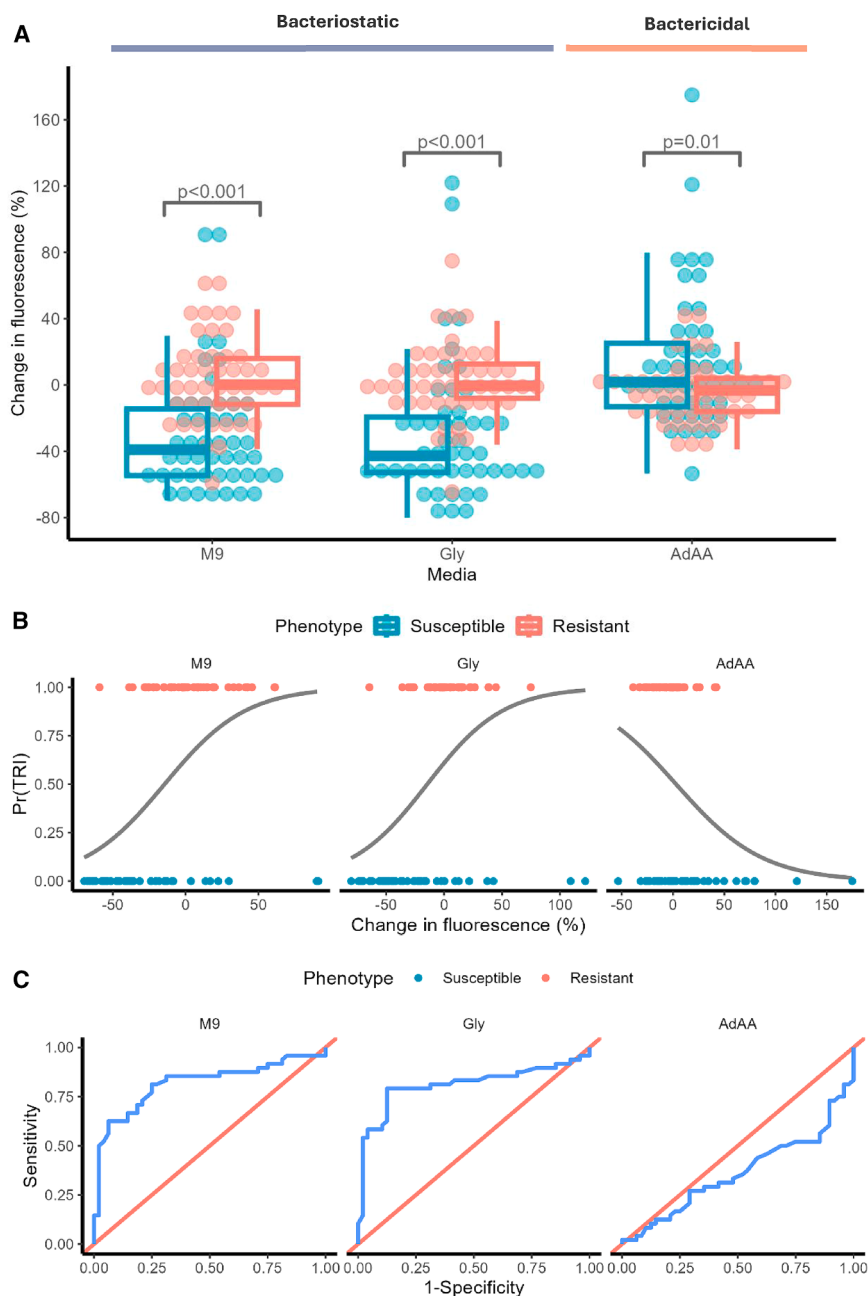


Figure 6. Measuring change in bacterial ATP following 30 min of trimethoprim exposure in M9 media and with supplementations of glycine (Gly) or adenine, glycine, and methionine (AdAA), comparing trimethoprim susceptible and resistant populations

(A) Boxplot illustrates the mean percentage change ($n = 4$) in intracellular ATP between an untreated control and a treated sample for 96 clinical UPEC isolates (48:48 susceptible [S] to resistant [R]) after 30 min of exposure to trimethoprim. This was performed in triplicate for the same media conditions as the time-course assay. Significance determined by binomial logistic regression.

(B) Binomial logistic regression curve for probabilities of trimethoprim resistance, $\text{Pr}(\text{TRI})$, against percentage change in fluorescence between a treated and untreated sample by media condition. Gray line is predicted values.

(C) Receiver operating characteristic plot for (B) illustrates the true positive rate (TPR or sensitivity) and false positive rate (FPR) subtracted from 1 (specificity) over a range of cut-off values for differentiating trimethoprim susceptibility. The diagonal line represents the chance diagonal.

14.3% mean increase in ATP in the susceptible group compared to a 4.5% decline for resistant isolates ($p = 0.01$, CI [0.12, 0.05]).

Capacity of different trimethoprim resistance determinants in limiting ATP salvage

As resistance to trimethoprim was demonstrated to provide protection against energy exhaustion in bacteriostatic conditions (Figure 6), we sought to investigate whether different resistance determinants provided the same degree of protection (Figure S6). Given that bactericidal conditions did not lead to energy exhaustion in susceptible groups, we focused on the two

and no trimethoprim susceptible isolate carried a known resistance mechanism. The mean change in fluorescence in both media for 5/6 *dfrA* variants tested was within $\pm 10\%$, with the exception of *dfrA12*, which skewed toward a larger positive value.

DISCUSSION

An *E. coli* UTI89 GSM model of trimethoprim-induced constraint on purine biosynthesis supported the hypothesis of free ATP being used to restore the purine and nucleoside availability in bacteriostatic conditions. This was due to trimethoprim

disrupting the purine biosynthesis pathway, which is a major source of *de novo* ATP. In bactericidal conditions, the availability of a purine in the media negated this effect, and free ATP remained untouched. These findings match the observations from *in vivo* metabolomics and with other antibiotics.^{5,19,20}

The model supported that the availability of methionine restored its restricted synthesis and restored spermidine. However, the model does not exclude the likelihood of methionine's role as the protein synthesis initiator, in the form of formyl-methionyl-tRNA^{met}, being relevant to bactericidal activity. To better understand the flow of glycine through the network, a combination of mass flow graphs and the Leiden algorithm was used to divide the network into functional communities. This further divided the network into sets of reactions where there were notable changes between treatment groups, which enabled the identification of where glycine was being used. We found that when glycine is available in the environment, the network uses it to synthesize pyruvate, which could serve to produce ATP and offer protection against reactive oxygen species.²¹

The cause of the depletion in intracellular ATP for trimethoprim-susceptible *E. coli* in M9 minimal media challenged with trimethoprim could arguably be attributed to the bacteriostatic behavior of trimethoprim in this condition. With the arrest of DNA, RNA, and protein synthesis caused by trimethoprim, and without metabolites available to compensate for the effect, the demand for ATP would decline. This agrees with the observations in *E. coli* by Lobritz et al.²² whereby bacteriostatic antibiotics slow down cellular respiration within 10 min of exposure. Nonetheless, the drop in intracellular ATP observed here and by Kwon et al.⁵ suggest cellular arrest of catabolic processes, which in turn slows down oxidative phosphorylation. This hypothesis is contradicted by an observation from Stepanek et al.²³ who demonstrated that *Bacillus subtilis* grown on Belitzky minimal medium supplemented with at least 10 mM ATP can tolerate 20-fold higher concentrations of trimethoprim compared to without ATP supplementation. Likewise, accumulations of adenine-based metabolites, such as ATP, at sites of infection reduce the lethal effects of antibiotics.²⁴ These observations suggest bacteriostatic trimethoprim activity leads to an ATP-expensive scenario rather than just cellular arrest. There is a growing body of evidence suggesting that purines, including phosphorylated purines such as ATP, are depleted by trimethoprim's bacteriostatic activity and that respiration is not the cause of the observed effect.^{5,23,25} A recent GSM modeling study by Yang et al.¹⁹ explored how antibiotics affect cellular energy with experimental validation. They provided evidence to suggest that purine limitation caused by trimethoprim challenge, similar to mutations in purine biosynthesis genes, increased ATP demand to restore the nucleoside pool. Indeed, models of this behavior suggested that adenine supplementation dramatically reduced the ATP demand. This agrees with the observation by Kwon et al.⁵ that purine supplementation did not lead to nucleoside depletion from trimethoprim challenge. The hypothesis of using ATP to restore nucleoside imbalance agrees with the observations previously mentioned, and importantly, does not contradict the observations of arrested DNA, RNA, and protein synthesis. Therefore, it would be logical to assume that the

means of improving the assay's performance is by ensuring there is a demand for nucleosides. This would explain why no depletion of intracellular ATP is observed in bactericidal trimethoprim conditions, as the nucleoside pool is compensated by a purine. Previous observations suggested glycine supplementation could improve ATP decline during trimethoprim challenge, but we observed no improvement over base M9 media. This suggests that the results reported by Kwon et al.⁵ in *E. coli* K-12 NCM3722 are not representative of *E. coli* and that the conclusion of glycine being the first metabolite to deplete after trimethoprim exposure is perhaps not the case for most *E. coli*.

Limitations of the study

The possibility of ATP being used for functions not considered in the model (e.g., motility, stress response, and repair) cannot be excluded. The synthesis of metabolites identified here as inhibited by trimethoprim agrees with the metabolomics observations of Kwon et al.⁵ However, the approach used here could not determine if metabolites accumulated or depleted, so only metabolites that directly involved folates for biosynthesis were identified. For example, our results suggested trimethoprim inhibits the synthesis of co-factors NAD and FAD in bacteriostatic conditions, but there are no known reports of this *in vivo*. It is possible that any effects on the availability of NAD and FAD require multiple generations, by which time the stalling of DNA, RNA, and protein biosynthesis prohibits cellular replication. However, this may not be the case for sulfonamides, given that bacteria require multiple generations before sulfonamides have an effect.^{8,26}

In conclusion, the results presented here provide strong evidence that bacteriostatic trimethoprim activity disrupts purine biosynthesis, with free ATP being salvaged to alleviate trimethoprim's actions.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Cailean Carter (cailean.carter@quadram.ac.uk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The GSM model data have been deposited at BioModels as MODEL2507240001 and are publicly available as of the date of publication.
- Code has been deposited at Zenodo and is publicly available as of the date of publication at <https://doi.org/10.5281/zenodo.16412170>.

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AUTHOR CONTRIBUTIONS

C.C. – conceptualization, formal analysis, investigation, methodology, software, validation, visualization, writing – original draft, and writing – review and editing. D.S. – writing – review and editing and supervision. J.W. – writing – review and editing and supervision. G.L. – conceptualization, review and editing, supervision, and funding acquisition.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the author(s) used Gemini for grammar and condensing information. After using this tool or service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Uropathogenic <i>Escherichia coli</i>	Carter et al. ²⁷	PRJEB60854 (European Nucleotide Archive)
Chemicals, peptides, and recombinant proteins		
Trimethoprim	Sigma-Aldrich	SBR0024
M9 minimal media	Formedium	MMS0101
Adenine hemisulphate salt	Sigma	A9126-25G
Glycine	Fisher Scientific	G/0800/60
L-methionine	Sigma	M-9625
Critical commercial assays		
BacTiter-Glo	Promega	G8231
Deposited data		
Metabolic model	BioModels	BioModels: MODEL2507240001
Code	Zenodo	https://doi.org/10.5281/zenodo.16412170
Software and algorithms		
ScrumPy	GitLab	https://gitlab.com/MarkPoolman/scrumpy

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Strains and conditions

One hundred and ninety-nine clinical *E. coli* isolates from urine have previously been characterized with a further 18 collected for this study in 2020 using the same methodology, which were all collected from Norfolk & Norwich University Hospital.²⁷ M9 minimal media (Formedium; MMS0101) was supplemented with 65 µg/mL adenine hemisulphate salt (Sigma), 50 µg/mL glycine (Fisher Scientific; G/0800/60), and 50 µg/mL L-methionine (Sigma; M-9625) as required. For all assays, isolates were grown in 1 mL LB broth overnight at 37°C in a standing incubator.

Data analysis and visualisation

All data analysis was performed using the Python programming language (v3.8.10) with data handling on Jupyter Notebooks with the pandas (v1.1.3) and NumPy (v1.18.3) packages. All code is available on GitHub (github.com/CaileanCarter/Metabolic-modelling-trimethoprim-action). Euclidean and Cosine distances were performed using their respective method from the NumPy spatial distances module. All plots were drawn with ggplot2 (v3.4.2) using the R programming language (v4.2.1) with significance values plotted using ggsignif (v0.6.4) and color scheme provided by ggsci (v3.0.0).²⁸ Radar charts were plotted with the fmsb package (v0.7.5).

The metabolic network maps were created with the Escher Python package (v1.7.3).²⁹ The ScrumPy *E. coli* UT189 model SBML file was converted into JSON format and all reaction and metabolite ids that were assigned a numerical value were changed to match the object's name. The network map was drawn for the purpose of this study to encompass glycolysis, TCA cycle, pentose phosphate pathway, purine biosynthesis, purine salvage, one carbon pool by folate, electron transport chain, oxidative phosphorylation, and maintenance ATP import/export. It should be noted that reversibility data were lost in converting formats and all reactions are shown as reversible.

METHOD DETAILS

Genome-scale metabolic modeling

Genome-scale metabolic (GSM) modeling was performed using ScrumPy3 (v3.0-alpha) and run on an Ubuntu 20.04 LTS on Windows Subsystem for Linux 2. An existing GSM model of *E. coli* UT189 was used and is available on BioModels (MODEL2507240001). Model properties are described in the [supplemental information](#) (Model properties and validation).

The media used for this model was M9 minimal media with individual transporters added for glucose, ammonium, sulfate, inorganic phosphate, water, oxygen, chlorine, and sodium. A list of biomass components produced by *E. coli* was derived from Feist et al.³⁰

which lists these components in proportion to each other. An exporting transporter was added for each biomass component. And the proportions were multiplied by the growth rate to obtain the flux values for biomass transporters (Table S1). For incorporating the metabolites supplemented to M9, constraints were added (when required) on the import of adenine, glycine, and methionine depending on the media condition being investigated. Where appropriate, flux bounds were applied to the import of these metabolites which represented the mM units of each that was supplemented to M9 minimal media. The media dependent constraints are outlined in Table 1.

Table 1. Constraints applied to import flux on media transporters for each media condition, i.e., M9 minimal media with/without metabolite supplementation

Metabolite	M9	+Gly	+Ad	+AdAA	+Thy	Flux bounds (upper, lower)
Adenine	0	0	≥ 0	≥ 0	0	37, 0
Glucose	≥ 0	≥ 0	≥ 0	≥ 0	≥ 0	11, 0
Glycine	0	≥ 0	0	≥ 0	0	66, 0
Methionine	0	0	0	≥ 0	0	33.5, 0
Thymidine	0	0	0	0	≥ 0	NA

Metabolites where import was always unrestricted

Ammonium	
Chlorine	
Pi	
Sodium	≥ 0
Sulfate	
Water	

For analyses that required flux bounds, the upper and lower flux bounds on the import flux of that metabolite were used. Highlighted cells indicate metabolites available under each media condition.

Gly, glycine; Ad, adenine; AdAA, adenine+glycine+methionine; Thy, thymidine; NA, flux bounds were not used for thymidine; Pi, inorganic phosphate.

Folate associated reactions

A set of reactions concerning the folate pool was defined by identifying any reactions involving folate metabolites DHF, THF, 5-methyl THF, 5,10-methylene THF, 5,10-methenyl THF, or 10-formyl THF from the stoichiometry matrix of the *E. coli* UT189 GSM (Table S5). The alternative formylated state of folate, 5-formyl-THF, was excluded as it is considered a storage form of folate and is not known to be a co-factor in any reaction (according to EcoCyc v27.1; accessed 03/03/2023). The biomass transporter for 5-methyl THF (MeTHF_bm_tx) was also excluded.

Defining metabolic pathways

To ensure modularity and simplicity, the reactions most relevant to this project such as reaction involved in folate metabolism, electron transport chain (ETC), tricarboxylic acid (TCA), and purine metabolism were categorized into five distinct groups, as detailed in Table S4. The metabolic network map for these reactions is illustrated in Figure S3.

Flux balance analysis

The methodology used here was as described previously.³¹ Briefly, flux balance analysis (FBA) was used for all metabolic modeling with minimisation of absolute total flux as the objective and steady state constraint ($N \cdot v = 0$). N was a stoichiometry matrix of the *E. coli* UT189 GSM with m rows for metabolites, n columns for reactions, and contents were the stoichiometries for each reaction. Further, v represented a vector of unknown flux values. Likewise, a weighting (ω) factor was sometimes changed for select reactions from the default value of 1 to confer a greater enzyme cost. Therefore, minimisation of the equation $\sum_{i=1}^n |v_i \cdot \omega_i|$.

Identifying folate-dependency for biomass components

$$\begin{aligned} \text{Minimize : } & \sum_{i=1}^n |v_i \cdot \omega_i| \\ \text{Subject to : } & \begin{cases} N \cdot v = 0 \\ v_{\text{Biomass component}} = -1 \end{cases} \end{aligned} \quad (\text{Equation 1})$$

The production of each biomass component (Table S1) in the GSM was confirmed with a feasible linear programming (LP) solution producing one relative unit of said metabolite (Equation 1) and reported as absolute total flux of the solution (which was also the objective value).

Absolute total flux of folates was calculated by summation of flux through folate associated reactions (Table S5) and provided a positive assertion that the synthesis of a metabolite utilized folates. The dependency on folates was determined using Equation 1 with the added constraint of setting flux for all folate associated reactions (Table S5) to zero while producing one unit of biomass component. The biomass component was deemed folate dependent if no feasible solution was made. To identify the reaction(s) within the folate pool used for the metabolite's synthesis, a constraint of zero flux was added to Equation 1 for the synthesis of THF (DHFR), methionine (methionine synthase), purines (phosphoribosylglycinamide formyltransferase and AICAR transformylase), or 2-ketovaline (3-methyl-2-oxobutanoate hydroxymethyltransferase).

Counteraction of trimethoprim-induced constraint on biomass synthesis by media supplementation

Counteraction by media supplementation was performed by permitting the import of adenine, glycine, methionine, and/or thymidine (Table 1) while all folate associated reactions (Table S5) were set to flux of zero as shown below:

$$\begin{aligned} \text{Minimize : } & \sum_{i=1}^n |v_i \cdot \omega_i| \\ \text{Subject to : } & \begin{cases} N \cdot v = 0 \\ v_{\text{Biomass component}} = -1 \\ v_{\text{Folate reactions}} = 0 \end{cases} \end{aligned} \quad (\text{Equation 2})$$

Network response to increasing dGTP demand under trimethoprim-induced purine biosynthesis inhibition

To investigate how metabolite supplementation could alleviate trimethoprim-induced purine biosynthesis inhibition, a series of LP formulations were solved repeatedly with increasing flux for dGTP export for each media condition. Uniquely to this analysis, adenine alone was included as a supplementation condition. The LP formulation was made as described in Equation 3 and relevant media condition flux bounds constraints (Table 1) were applied (including glucose flux bounds). Arbitrary upper flux constraints were applied to two purine biosynthesis reactions involving folates, GAR formyltransferase and AICAR transformylase, to simulate trimethoprim restriction on purine biosynthesis. Export flux of dGTP was incremented to the maximum arbitrary value of 20; this upper bound needed to be greater than the bounds placed on purine biosynthesis but not so large that most of the output had no change in reaction sets. Limitations were placed on ATP salvage by restricting ATPase to a flux of zero.

$$\begin{aligned} \text{Minimize : } & \sum_{i=1}^n |v_i \cdot \omega_i| \\ \text{Subject to : } & \begin{cases} N \cdot v = 0 \\ v_{\text{dGTP export}} = -A; 0.05 \leq A \leq 20 \\ 0 \leq v_{\text{GAR - formyltransferase}} \leq 2 \\ 0 \leq v_{\text{AICAR - transformylase}} \leq 2 \\ v_{\text{ATPase}} = 0 \end{cases} \end{aligned} \quad (\text{Equation 3})$$

ATP bioluminescence time-course assay

250 μL of overnight culture of a trimethoprim susceptible UPEC isolate was transferred to 25 mL pre-warmed media in a 250 mL baffled flask and incubated at 37°C, shaking at 140 rpm till an OD₆₀₀ of ~ 0.3 . When solely using M9 without supplementation, 1 mL of overnight was transferred to 24 mL M9 to reduce the time taken to reach OD₆₀₀ of ~ 0.3 . From the baffled flask, cells were diluted to 0.02 OD₆₀₀ in 8 mL pre-warmed M9 media (supplemented when required) and vortexed thoroughly. 80 μL of diluted culture was transferred to each well of a 96-well Thermo Scientific Nunc optic clear bottom white walled plate. The assay was performed on the CLARIOstar Plus (BMG Labtech) with two injectors using a custom script provided by BMG Labtech which was further modified to meet the experimental design described here. One injector was primed with BacTiter-Glo reagent (Promega) and prepared according to manufacturer's specifications. The CLARIOstar Plus' environmental control was set to 37°C for the duration of the assay. Trimethoprim (Sigma-Aldrich; SBR0024) was prepared at 9X the desired concentration in pre-warmed M9 media (supplemented when required). The assay began with a 5-min incubation period before measuring OD₆₀₀ across the whole plate every 15 min for three measurements. Each column of the plate was treated as a timepoint in the assay. Following the three baseline absorbance measurements, three ATP bioluminescence baseline measurements were taken. For each timepoint, the OD₆₀₀ was measured first before injecting an equal volume in the well of BacTiter-Glo reagent and shaking at 100 rpm for a minute, and measuring ATP

bioluminescence using the Ultra-Glo pre-set. The plate was then ejected and 10 μ L of pre-warmed media (control) or antibiotic (treated at 36 mg/L for a final concentration of 4 mg/mL) solution was added to the appropriate wells and inserted back into the machine. The remainder of 15 min was waited for. Thereon, ATP bioluminescence readings were taken every 10 min for nine measurements. All time-course assays were repeated in triplicate.

Incorporating experimental data to model the salvage of free ATP under trimethoprim stress

To better understand how free ATP would be used under trimethoprim challenge, experimental data were incorporated into the model whereby the network would synthesize free ATP and a portion of this could later be salvaged under trimethoprim challenge. Although *in vivo* free ATP would be available intracellularly within the population of cells as the population grew, *in silico* FBA under steady state does not permit accumulation of intracellular metabolites (hence biomass components are treated as external metabolites). Therefore, an external ATP pool was introduced that could be salvaged by the network under conditions of stress. This form of ATP was termed maintenance ATP (mATP) to differentiate it from the biomass ATP which was a component of RNA which hereon is referred to as biomass ATP (Table S2).

Converting optical density to cell counts for growth rate

The absorbance (OD) data were converted into total number of cells (n_{cells}) for each value at each time point (t) using the formula described in Equation 4. Absorbance was converted to total cell counts for two reasons: a) there was a change in well volume during the assay (addition of media/trimethoprim) which increased the distance light needed to travel through the liquid which alters OD and b) the growth rate derived from absorbance did not scale biomass export into the same range as the ATP bioluminescence measurements. Total cell count was calculated with a conversion factor from Volkmer and Heinemann³² for *E. coli* in M9 minimal media with glucose supplementation (Equation 4). This conversion factor was multiplied by the volume (V) of the well and OD.

$$n_{cells} = m \cdot V \cdot OD$$

$$m = 11.1 \cdot 10^8 \text{ cells} \cdot \text{mL}^{-1} \cdot \text{OD}^{-1} (\text{conversion factor})$$

$$V = \begin{cases} 0.08 \text{ mL, if } t \leq 75 \\ 0.09 \text{ mL, else} \end{cases} \quad (\text{Equation 4})$$

Linear regression

The cell numbers and ATP bioluminescence data from the time-course assays were used to provide the growth rate and total ATP for the metabolic model for each media and treatment condition. A linear regression was made using the Python package statsmodels' *ols* function (v0.12.2) and included baseline measurements from control groups. Baseline measurements for treated groups were excluded from the models as control groups would be used instead. Separate linear regressions were made for cell counts and ATP bioluminescence (FLU), and each included time, media type, and treatment as predictive variables with interactions between them (R formula: $[n_{cells}|FLU] \sim \text{time} * C(\text{treatment}) * C(\text{media})$). Assumption were checked using the Python package Seaborn *lmplot* for linearity, a qqplot to assert data were normally distributed, and a stem plot of Cook's distance to assert no data points had undue influence over the model. The growth rate (μ) was calculated for each media and treatment group from the linear regression; the slope of the line from the cell count model was multiplied by a constant factor of 0.1 from Díaz Calvo et al.³³ which enabled comparisons between OD and growth rate.

Total free ATP, maintenance ATP, and biomass ATP

A maintenance cost normally covers the energy required to maintain basic cell functions like cell membrane integrity, polymerisation of proteins, and repairing DNA, however, the maintenance costs were unknown for the different media and treatment conditions. Further, it was suspected that this maintenance cost would be dramatically different between these different conditions.³⁴ Therefore, experimental values were used to capture the total free ATP which included mATP. Although it was suspected that the demand for RNA and DNA components would change immediately after trimethoprim challenge, experimental data was not available. Thus, the demand for these components immediately after trimethoprim-like conditions were the same as control conditions, which was proportional to the growth rate.

Experimentally collected ATP bioluminescence data were used as an indicator of total free ATP present in the population for each treatment and media condition. As there was no available conversion factor for converting relative light units (RLUs) derived from ATP bioluminescence to *in silico* total ATP, it was assumed that RLUs equaled the total ATP present in a sample. This was informed by the reaction stoichiometry for the luciferase reaction (Table S6) used for ATP bioluminescence; the net stoichiometry implied that *in vivo* total free ATP is directly proportional to the number of photons, measured as RLUs, which was measured *in vivo*.

The total free ATP captured by the luciferase reaction was assumed to capture both mATP and biomass ATP (but not ATP already incorporated into RNA). Experimental RNA synthesis rates were not collected, therefore, the rate of ATP produced for RNA in each media and treatment condition was the flux value of RNA ATP export (Table S1) multiplied by the growth rate (μ). Likewise, the rate of

change in total free ATP $\left(\frac{dATP_{Total}}{dt}\right)$ for each media and treatment condition was the slope of the linear regression line. Therefore, the unknown mATP cost was calculated by subtracting the biomass ATP production rate from the total free ATP production rate.

Defining a salvageable external maintenance ATP pool

To observe whether the model would salvage mATP under trimethoprim stress in the different media conditions, a pool of external mATP was made available to the model. Like *in vivo*, the model needed to create this pool of mATP as the population grew under normal growth conditions before trimethoprim stress was evoked. Therefore, the quantity of mATP in a sample was proportional to the population size. However, as biomass ATP and mATP were unknown quantities, their rates of change were used to determine proportions as described in Equation 6. Therefore, the relative units (RU) of mATP change over time could act as a proxy for the availability of mATP for salvage. Also, flux values for total ATP were used rather than absolute values to see how mATP was produced and used under trimethoprim stress, rather than telling the model how much mATP needed to be salvaged.

$$\begin{aligned} \frac{dATP_{Total}}{dt} &= V_{Biomass\ ATP\ export} + V_{mATP\ export} \\ V_{Biomass\ ATP\ export} &= \mu \cdot \frac{dATP_{RNA}}{dt} \end{aligned} \quad (\text{Equation 6})$$

To assert there would be sufficient demand for ATP relative to the mATP available, a series of calculations were solved to find the proportions of biomass ATP and mATP at each time point from the ATP time-course assays (Equation 7). These calculations did not require FBA as internal flux values were not needed for this analysis. Because the assay measurements began at the 30-min point, 1 min was used as the time difference at the 30-min mark. Baseline measurements for the treated group were from the control group as the linear regression excluded baseline measurements as it would not have passed the linearity assumption.

$$\begin{aligned} \text{Total free ATP at } t_n &= \sum_{i=30}^n \frac{dATP_{Total}^G}{dt} \cdot (t_i - t_{i-1}) \\ \text{Total biomass ATP at } t_n &= \sum_{i=30}^n V_{Biomass\ ATP\ export}^G \cdot (t_i - t_{i-1}) \\ \text{Total mATP at } t_n &= \text{total free ATP at } t_n - \text{total biomass ATP at } t_n \\ \text{Treatment group} &= G \begin{cases} \text{Control, if group is control} & | \ t_n \leq 60 \\ \text{Treated, else} \end{cases} \end{aligned} \quad (\text{Equation 7})$$

Optimum weighting for mATP import and folate associated reactions

To be confident that the model would not import mATP as a free energy source, an optimum weighting was required for the mATP import reaction (mATP_tx) which would prevent the model importing mATP under normal growth conditions. A series of LP solutions with the base formulation in Equation 8 were calculated with increased weighting applied to mATP import. This was done for each media condition as media supplementation meant different energy requirements, therefore, different optimum weightings. The optimum weighting was determined to be the minimum mATP_tx weighting whereby flux through mATP_tx was zero.

$$\begin{aligned} \text{Minimize : } & \sum_{i=1}^n |v_i \cdot \omega_i| \\ \text{Subject to : } & \begin{cases} N \cdot v = 0 \\ V_{mATP\ import} \cdot \omega_i; \ 1 \leq \omega_i \leq 120 \\ V_{All\ biomass} = fd \cdot \mu \\ V_{mATP\ export} = \frac{dATP_{Total}}{dt} - V_{Biomass\ ATP\ export} \end{cases} \end{aligned} \quad (\text{Equation 8})$$

To simulate trimethoprim conditions, an optimum weighting was required to be applied to all folate associated reactions (Table S5), for all media conditions (Table 1), that would result in the import of mATP. This was performed similarly to mATP_tx and included media-specific optimum weighting for mATP import (ω_i^*) as determined in Equation 8. The optimum weighting for folates was deemed to be the minimum weighting on folate associated reactions which achieved the maximum import flux of mATP. A summary LP formulation is provided in Equation 9.

$$\begin{aligned} \text{Minimize : } & \sum_{i=1}^n |v_i \cdot \omega_i| \\ \text{Subject to : } & \begin{cases} N \cdot v = 0 \\ v_{\text{Folate reactions}} \cdot \omega_i; 1 \leq \omega_i \leq 120 \\ v_{\text{mATP import}} \cdot \omega_i^* \\ v_{\text{All biomass}} = fd \cdot \mu \\ v_{\text{mATP export}} = \frac{dATP_{\text{Total}}}{dt} - v_{\text{Biomass ATP export}} \end{cases} \end{aligned} \quad (\text{Equation 9})$$

In-silico time-course ATP assay

The *in-silico* ATP time-course assays were simulated by enabling the model to salvage external mATP. Because the LP formulation for this analysis required some mATP to be available at the start, *in vivo* data were used instead of an arbitrary number as it was not possible to do a zero division. Thus, prior to the first time point (<30 min) the availability of mATP was calculated using Equation 7 with the addition of the intercept from the ATP bioluminescence linear regression for each condition. For example, to calculate the total free ATP before baseline measurements:

$$\text{Total free ATP at } t_{29} = \frac{dATP_{\text{Total}}}{dt} \cdot 29 + ATP_{\text{intercept}} \quad (\text{Equation 10})$$

From thereon, a series of LP formulations were solved at each time point (t). The import of mATP was constrained to the total accumulated mATP divided by Δt to prevent the solution from importing more mATP than was available in the external mATP pool. Constraints applied during baseline time points for the treated group were the same as the control group which included growth rate and weighting applied to folate associated reactions. At the 60-min mark, the weighting to folate associated reactions was applied to the treated group, however, the control group's growth rate was used until the 75-min mark to simulate the demand for ATP. This is summarized in the following LP formulation:

$$\begin{aligned} \text{Minimize : } & \sum_{i=1}^n |v_i \cdot \omega_i| \\ \text{Subject to : } & \begin{cases} N \cdot v = 0 \\ v_{\text{All biomass}} = fd \cdot \mu; \mu = \begin{cases} \mu_{\text{Control}}, & \text{if control} \mid t \leq 75, \\ \mu_{\text{Treated}}, & \text{else} \end{cases} \\ v_{\text{mATP export}} = \frac{dATP_{\text{Total}}}{dt} - v_{\text{Biomass ATP export}} \\ v_{\text{Folate reactions}} \cdot \omega_i; \omega_i = \begin{cases} 10; & \text{if treated} \wedge t \geq 75, \\ 1; & \text{else} \end{cases} \\ 0 \leq v_{\text{mATP import}} \cdot \omega_i^* \leq \left(\frac{\text{total mATP}}{\Delta t} \right); \Delta t = \begin{cases} 15, & \text{if } t \leq 75, \\ 10, & \text{else} \end{cases} \end{cases} \end{aligned} \quad (\text{Equation 11})$$

The total external ATP was calculated at each time point using Equation 12 with the division of biomass ATP and maintenance ATP following the principle in Equation 6.

$$\begin{aligned} \text{Total free ATP at } t_n &= \sum_{i=30}^n \left(v_{\text{Biomass ATP export}} + \frac{dATP_m}{dt} \right) \cdot (t_i - t_{i-1}) \\ \frac{dATP_m}{dt} &= v_{\text{mATP export}} - v_{\text{mATP import}} \end{aligned} \quad (\text{Equation 12})$$

The centrality of reactions of reactions was calculated using PageRank (*PR*) with a dampening factor of 0.85.³⁵

Mass flow graphs and functional groups

For clustering reactions into functional groups to understand glycine's role in bactericidal trimethoprim action, mass flow graphs were made using a modified approach from Beguerisse-Díaz et al.³⁶ For each media and treatment group permutation, the stoichiometry matrix (N) was filtered for reactions and metabolites present in the LP solution. The consumption and production of each metabolite per reaction in N was calculated by multiplying the respective flux value (v) from the LP solution by the column (n), giving $N(v)$. An adjacency matrix (A_{ij}) was made from $N(v)$. For calculating the edges, the flow of a metabolite ($\frac{dS_k}{dt}$) from a producing reaction (R_i) to a consuming reaction (R_j) was calculated using an adjusted equation from Beguerisse-Díaz et al.³⁶ as shown below:

$$\frac{dS_k}{dt} \text{ from } R_i \text{ to } R_j = \frac{dS_{ki}^+}{dt} \cdot \left(\frac{dS_{kj}^-}{dt} / \sum \frac{dS_k^-}{dt} \right) \text{ where } \begin{cases} S_{ki}^+, \text{ metabolite produced by } R_i, \\ S_{kj}^-, \text{ metabolite consumed by } R_j \end{cases} \quad (\text{Equation 13})$$

The summation of Equation 13 across all metabolites connected between R_i and R_j were used as weighting (ω) for the edges in A_{ij} :

$$A_{ij}(\omega) = \sum_{k=1}^n \frac{S_{ki}^+}{dt} \cdot \left(\frac{S_{kj}^-}{dt} / \sum \frac{S_k^-}{dt} \right) \quad (\text{Equation 14})$$

Mass flow graphs were drawn with igraph (v0.10.6) in Python using the edges determined above to draw connections between metabolites with mass flow as a weighted attribute. The shared community structure was identified using the Leiden algorithm Python package (v0.10.1) with the `find_partition_multiplex` method and arguments ModularityVertexPartition, number of iterations of -1 for the algorithm to run until no improvement was made, seed of 1, and weights were set to the edge weights.³⁷

High-throughput ATP bioluminescence screening

Overnight cultures were performed in clear 96-well plates with 300 μ L LB broth which were inoculated from frozen stocks. 8 μ L of overnight culture were transferred to two wells with 72 μ L of M9 media (supplemented when required) in white 96-well plates (Thermo Scientific). Cells were left to acclimate for 10 min at 37°C. 10 μ L of M9 media (supplemented when required) was added to control wells and 10 μ L 36 mg/L trimethoprim was added to treated wells for a final concentration of 4 mg/L and incubated at 37°C for 30 min. ATP bioluminescence was measured using the CLARIOstar Plus. For this, the plate reader injected 90 μ L of BacTiter-Glo to each well followed by shaking at 100 rpm for 20 s. Fluorescence was measured at 550-570 nm 1 min after injection. The change in fluorescence between untreated and treated wells was calculated by $RLU_{treated}/RLU_{untreated}$ and taking the mean across triplicates.

QUANTIFICATION AND STATISTICAL ANALYSIS

Linear regression for time-course ATP bioluminescence

A linear regression was used to provide growth rate and total ATP for the model using statsmodels' `ols` function to fit $[n_{cells}|FLU] \sim time * C(treatment) * C(media)$ as described previously. Assumptions were checked using the Python package Seaborn `lmlplot` for linearity, a qqplot to assert data were normally distributed, and a stem plot of Cook's distance to assert no data points had undue influence over the model.

Statistical analysis for ATP bioluminescence screening

A binomial logistic regression was used to determine whether the change in fluorescence between treated and untreated in trimethoprim susceptible and resistant samples were statistically significant in each media condition. This was performed using the `glm` method from statsmodels (using binomial family) with change in fluorescence as the predictor variable and trimethoprim susceptibility as the endogenous variable. The linearity between the predictor variable and logit was checked before calculating the log odds. A stem plot of Cook's distance was also used to assert no data points had undue influence over the model. Receiver operating characteristics (ROC) were performed by iterating over a series of threshold values between 1% and 300%. Optimal cut-off values for distinguishing susceptibility were identified by maximum Youden's index.

Variability in ATP between ATP bioluminescence screening

For comparing deviation from the sampling mean, the change in fluorescence for each replicate of an isolate was adjusted by subtracting the isolate mean: $x_i - \bar{x}_i$ whereby x_i was the change in fluorescence for each replicate measurement for each isolate, $\bar{x}_i$ was the mean change in fluorescence for the isolate x_i . Therefore, the overall population mean was zero – allowing straightforward comparison in deviation from the mean between isolates. Two standard deviations ($\pm 2\sigma$) of all adjusted data points within a group were then calculated.