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The *in vitro* redundant enzymes PurN and PurT are both essential for systemic infection of mice in *Salmonella enterica* serovar Typhimurium

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Abstract

Metabolic enzymes show a high degree of redundancy, and for that reason they are generally ignored when searching for novel targets for anti-infective substances. The enzymes PurN and PurT are redundant *in vitro* in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), where they perform the third step in the purine synthesis. Surprisingly the results of the current study demonstrated that single gene deletions of each of the genes encoding these enzymes caused attenuation (competitive infection index < 0.03) in mouse infections. While the Δ*purT* mutant multiplied as fast as the wild type strain in cultured J774A.1 macrophages, net multiplication of the Δ*purN* mutant was reduced by approximately 50 % in 20 hours. The attenuation of the Δ*purT* mutant was abolished by simultaneous removal of the enzyme PurU, responsible for formation of formate, indicating that the attenuation was related to formate accumulation or wasteful consumption of formyl-tetrahydrofolate by PurU. In the process of further characterization, we disclosed that *in vivo* the enzyme-complex GCV was the most important for formation of C-1 units *in vivo* (CI: 0.03 ± 0.03). In contrast, GlyA was the only important enzyme for the formation of C-1 units *in vitro*. The results with the Δ*gcvT* mutant further revealed that formation of serine by SerA and further conversion of serine into C-1 units and glycine by GlyA was not sufficient to ensure C-1 formation in *S.* Typhimurium *in vivo.* The study calls for re-investigations of the concept of metabolic redundancy in *S.* Typhimurium *in vivo.*

Introduction

Salmonella enterica is a common cause of food borne disease worldwide. Annually, more than 93 million people have been estimated to suffer from non-Typhoid salmonellosis, and more than 155.000 people succumb to the disease (1). Infection with the serovar *S.* Typhimurium in mice does not cause diarrhoea, but results in a systemic, life-threatening condition, where bacteria predominantly localize in cells of the immune system in the liver and spleen. For this reason, *S.* Typhimurium infection of mice is used as a model for systemic salmonellosis, including infection with the host-specific serovar *S.* Typhi (2).

The diversity of intra-cellular and extra-cellular host-niches occupied by *S.* Typhimurium is reflected in a high-degree of metabolic flexibility (3). This flexibility is achieved through component and systems-level redundancy in the metabolic network (4), and recent years have seen several studies of *S.* Typhimurium in order to understand the importance of metabolic redundancy for its pathogenic life style (5-8). These studies have used genome scale metabolic modelling to predict essential and combined-lethal metabolic reactions; the latter group consists of two or more non-essential reactions, which, when considered as one unit, are found to be essential. Such combinations are referred to as minimal cut-sets in metabolic modelling (9).

A list of 102 cut-sets of metabolic reactions in *S.* Typhimurium was recently produced using a novel genome-scale metabolic model. Each cut-set was predicted to be essential for growth in a modified M9-minimal medium, and the underlying assumption was that blocking such combinations of reactions would attenuate *S.* Typhimurium during infection. One cut-set was the combination of reactions carried out by the enzymes PurN (glycineamide-ribonuclease-transformylase-N) and PurT (glycineamide-ribonuclease-transformylase-T) in the purine

biosynthesis pathway (8). A study using a similar approach by Thiele et al. (5) also predicted that combined blocking of PurN and PurT would be detrimental to growth.

Purine *de novo* synthesis consists of ten steps, converting phosporibosyl-pyrophosphate (PRPP) into inosine-monophosphate (IMP) (10). In protobacteria the third step, which converts 5´-phosphoribosyl-glycineamide (GAR) into formyl-phosphoribosyl-glycinamide (fGAR), is carried out by the two enzymes in the predicted cut-set, PurN and PurT, using different formyl donors. Blockage of this step results in accumulation of GAR and depletion of all down-stream products, why it is considered essential for the purine synthesis (11). The reactions carried out by PurN (GART-RXN) and PurT (GARTRASFORMYL2-RXN) are thus textbook examples of functional redundancy in metabolic reactions, and the prediction as a cut-set in *S.* Typhimurium was not surprising.

PurN and PurT are not isoenzymes because PurN, which is found in both prokaryotes and eukaryotes (10,12), obtains the formyl-group for generation of fGAR from formyl-tetrahydrofolate (fTHF), while the prokaryote-specific enzyme PurT uses formate (13) (Fig 1A). As seen in Fig 1B, PurN and PurT create a link between purine synthesis and the folate Carbon-1 (C-1) metabolism, where essential C-1 units are formed (14). Aside from delivering carbon-2 and carbon-8 in purine synthesis, the folate metabolism delivers methyl-groups for the amino acid methionine and the deoxynucleotide dTMP. PurT is unique in that it uses formate as C-1 donor. Under anaerobic conditions formate is formed from pyruvate in the oxygen sensitive pyruvate formate lyase (PFL) reaction (15), but under aerobic conditions, formate is solely derived from fTHF in a reaction catalysed by the PurU enzyme (16) (Fig 1A). If the PurT enzyme does not use formate, it is a dead-end product in aerobic metabolism, and it cannot be fuelled back into the C-1 metabolism (17). Like in *E. coli* (14,18), C-1 units for amino acid and purine synthesis are produced by the

GlyA reaction during conversion of serine into glycine, and from degradation of glycine to ammonia and carbon dioxide by the glycine cleavage system (GCV) (Fig 1B).

Mutants that lack *purN* or *purT,* or both genes, have been constructed and characterized in *E. coli* (11). *purN* and *purT* mutants grew slightly slower than their parental strains in minimal medium under both aerobic and anaerobic conditions. Interestingly, growth of the *purN* mutants was inhibited by addition of glycine under aerobic, but not under anaerobic conditions, suggesting that the effect of glycine was due to limiting formate production. A kinetic analysis of the purified PurU enzyme offered an explanation for this phenomenon, as its hydrolase activity was severely inhibited by glycine (18). In the *purN* mutant, glycine inhibition of formate production by PurU thus prevented fGAR synthesis by PurT. When the authors found the PurU activity to be activated also by histidine, they proposed that the PurU enzyme functions as a regulator that balances the folate intermediates tetrahydrofolate (THF), methylene tetrahydrofolate (mTHF), and formyl tetrahydrofolate (fTHF) as a function of the glycine and methionine concentrations.

Currently we lack good knowledge on the *in vivo* metabolism (in the host) of pathogenic bacteria, even though this arguably is just as important as virulence factors for the ability of the pathogenic bacterium to carry out the infection (19). This included lack of knowledge on which nutrients the bacteria can scavenge from the host, which metabolites different bacteria needs to synthesize and how this differs between different bacteria, different hosts and even different places the bacterium occupies in the same host during infection. In the absence of experimental data, we rely on deduction from their *in vitro* (laboratory) growth phenotypes and model simulations. In some situations we observe good correlations between *in vitro* and *in vivo* phenotypes with respect to prediction of the ability to carry out the infection (8), but sometimes this is not the case, probably because functional auxotrophy may arise when metabolites are present in levels below an

118 important threshold during infection of the host, resulting in unforeseen regulatory effects. Under
119 such conditions, predicted redundant reactions may turn out to be non-redundant. In the current
120 study, we show that the universally acknowledged redundant enzyme-pair, PurN/PurT does not
121 show functional redundancy in *S.* Typhimurium during infection of mice. Rather, each enzyme is by
122 itself essential for infection. Likewise the redundant enzyme pair MetE/MetH was shown to be non-
123 redundant during mouse infection. An important message from this study is that well-established
124 pairs of redundant enzymes may be functionally non-redundant *in vivo* and cannot *a priori* be
125 classified as redundant based upon metabolic modelling. So there is a dire need to reinvestigate
126 the concept of metabolic redundancy in *Salmonella* and other pathogenic bacteria in the *in vivo*
127 situation.
128

129 ## Material and Methods

130

131 ### Bacterial strains

132 *S.* Typhimurium 4/74 was used as wild-type strain and for the construction of mutant strains by
133 lambda red mediated re-combination (20), essentially as previously described (8) (Table 1).
134 Mutated alleles were transformed to a clean wild type *S.* Typhimurium 4/74 background by
135 P22HT105/int^{-}20 mediated transduction, as described (8). Transduction was also used to construct
136 double and triple mutants. For construction of triple mutants, the resistance marker, normally
137 kanamycin, was flipped out using the FLT system as described (20), while each gene contained a
138 different antibiotic cassette in the double mutants. Primers for mutant construction are listed in
139 Supplementary Material, Table S1, together with primers used to control the mutations, generally
140 by two PCR-reactions, one targeting the inserted antibiotic resistance gene and the flanking
141 regions of the desired genes and one targeting both flanking regions.
142

143 Genetic complementation was obtained by PCR-amplification of the relevant gene and subsequent
144 cloning into the low copy number plasmid pACYC177, followed by transformation of the resulting
145 plasmid into the mutant strain, essentially as described (21). Primers are listed in Supplementary
146 Material, Table S1. Restriction enzymes XhoI and BamHI were used for cloning according to the
147 manufacturer´s recommendation (Thermo Scientific, by, Denmark), and constructs were verified
148 by restriction analysis and sequence analysis.
149
150 **Culture and growth conditions**
151 Growth of bacteria in rich media was done in DifcoTM Lysogeny broth, Lennox (Becton, Dickinson
152 and Company, Albertslund, Denmark) and on LB agar plates (Becton, Dickinson and Company,
153 Albertslund, Denmark), and growth in minimal medium was carried out in M9-broth (2 mM MgSO4,
154 0.1 mM CaCl2, 0.4 % glucose, 8.5 mM NaCl, 42 mM Na2HPO4, 22 mM KH2PO4, 18.6 mM NH4Cl).
155 Chloramphenicol 10 ug/ml, kanamycin 50 ug/ml, glycine 0.55 mg/ml, serine 0.55 mg/ml and
156 methionine 0.55 mg/ml (Sigma, Denmark), were added when appropriate.
157
158 Growth phenotypes of mutants were determined by first growing the bacterium overnight in LB
159 flasks at 37 °C with shaking (200 rpm). This culture was diluted 40 fold into 0.2 ml M9 and growth
160 was followed for 24 hours at 37 °c with shaking (250 rpm) by OD_{600} measurement each 15
161 minutes in a BioScreen CTM format with biological triplicates and technical replicates. Wild type
162 strain and blank wells were included as controls. Growth curves were extracted using Excel
163 (Microsoft, SanDiego, USA) and OD_{600} values were corrected for the blank controls.
164
165 **Microscopic appearance of bacteria**
166 The microscopic appearance of bacteria was determined by phase contrast microscopy at fixed
167 time points and conditions using an AxioCamHR4 phase-contrast microscope. Three hundred

individual cells were observed to determine the most common cell morphology. Continuous observations of cell morphology during growth in LB media at 37 °C was done using oCelloScope bright field camera (magnification of approximately 200x and resolution of 1.3 µm) as reported (22).

Macrophage survival experiments

Survival and multiplication inside J774 macrophages were measured as described (21). *S.* Typhimurium 4/74 was used as reference wild type, and *ΔssaV* mutant in the 4/74 background (21) was used as negative control. Deletion of this gene renders *S.* Typhimurium incapable of intra cellular replication (23). Briefly, MOI of infection was 5 and 25 minutes was allowed for the initial uptake of bacteria, whereafter gentamicin (100 ug/ml) was added for one hour and then replaced with 25 ug/ml for the rest of the experiments. CFU counts with three or four biological repeats and with technical duplicates were obtained at the point of the first addition of gentamicin, and after 1 hour, 2 hours and 21 hours of incubation with this drug.

Mouse infections

Measurement of infection efficacy was performed using a systemic model of infection in C57/BL6 female mice (Taconic, Denmark). The competitive challenge model (24) where wild type and mutant strain were given together in the same mouse was used, as described (8). Briefly, mice were inoculated by the I.P. route with 0.1 ml of an approximately 1:1 mixture of wild type and mutant strains in PBS. The inoculum was standardized to contain a challenge dose of 5×10^3 bacteria of each strain using CFU measurements. The exact amount of each strain in the inoculum was determined by plating serial dilutions on LB plates. The ratio between wild type and mutant bacteria in the spleen was determined 6 days post-inoculation by plating dilution series on LB agar and subsequently determining the resistance (chloramphenicol or kanamycin resistance) of 100

colonies. Sensitive bacteria corresponded to wild type and resistant bacteria to mutants. The competitive index was calculated as the mutant/wild type ratio of the spleen count versus the mutant/wild type ratio of the inoculum. Severely affected mice were humanely killed. If the spleen of such mice contained $>10^5$ CFU *Salmonella* this was expected to be the cause of the disease, and colony counts of such mice were included in the competitive index scoring.

Ethical statement

Mice challenge experiments were conducted with permission to the senior author from the Danish Animal Experiments Inspectorate according to Danish by-law No. 474 of 15th May 2014 (license number: 2009/561–1675).

Statistical analysis

Statistical differences between wild type and mutant strains in CFU, and in virulence measured in mice, was determined using GraphPad Prism®, version 5.0 (GraphPad software, Town, USA) with one-sample t-test analysis. Grubb´s outlier test was performed to exclude outliers with a significance of 0.05.

Results

PurN and PurT are redundant *in vitro* but not *in vivo.*

PurN and PurT have been reported to be redundant for growth of *E. coli*, although *purN* and *purT* mutants grow somewhat slower than the wild type (11). Single mutants in *S.* Typhimurium 4/74 created by deletion of the *purN* and *purT* genes grew as well as the parental wild type strain in minimal medium (WT: μ= 0.37±0.017, Δ*purN*: μ= 0.35±0.01 and Δ*purT*: μ= 0.36±0.02), while

the double mutant did not grow. The growth determination of the Δ*purN* mutant is shown in Supplementary Figure S1 to demonstrate the way specific growth grates were determined. When grown in rich media (LB), the double mutant had no growth defect (data not shown). This corresponds to PurN and PurT being redundant for growth in minimal medium *in vitro*.

When the mutants were analysed for their virulence in mice, using competitive challenging experiments, both single and double mutants were severely attenuated, showing competitive indexes (CIs) below 0.03. Virulence of single mutants could be raised to normal levels by complementation with wild type genes *in trans* (Table 2), showing that the attenuation was due to lack of PurN and PurT, respectively. Thus, in the infection situation, one or more factors are limiting for growth in an enzyme-specific manner for the presumed redundant enzyme-pair PurN/PurT, clearly showing that they are not redundant *in vivo* during infection of mice.

Mutation of *purN* but not *purT* attenuates the strain during interaction with cultured macrophages.

Interaction with macrophages is an important step in the development of systemic salmonellosis in mice, and the majority of mutants that fail to grow in cultured macrophages have turned out to be attenuated during mice infection (25). To investigate the ability to grow in macrophages, we challenged cultured J774A.1 macrophages with Δ*purN* and Δ*purT* mutants. As seen in Table 2, the WT strain was found to multiply 9.7± 2.5 times in 20 hours. This equals approximately seven-hour generation time, and corresponds to estimates for multiplication of *S.* Typhimurium in the spleen of mice *in vivo* (26). In contrast, the Δ*purN* mutant only multiplied 4.4 ± 3.5 times, which was significantly less than the wild type strain (p=0.03). The Δ*purT* mutant resembled the wild type strain and multiplied 8.0 ± 5.6 times, suggesting that the attenuation in virulence caused by the deletion of *purT* was not related to interaction at the macrophage level, while attenuation of the

242 *ΔpurN* mutant might be related to replication in cells of this type. The double mutant was severely
243 attenuated, and the resulting number of bacteria after 20 hours was reduced 70% compared to
244 the number of cells taken up by the macrophages (Table 2). The control strain, *S.* Typhimurium
245 4/74 lacking *ssaV*, encoding an effector protein of the type three secretion system, SPI-2,
246 associated with intracellular multiplication (23), multiplied 2.1 ± 0.6 times, which was within the
247 expected range, showing that the assay was performing as expected. The differences in
248 multiplication were not caused by different starting concentrations, since all strains were taken up
249 by the macrophages to the same degree (data not shown).
250
251 **Cooperation between PurT and PurU *in vitro*, in macrophages and during mouse**
252 **infection**
253
254 Conversion of GAR to fGAR by the PurT enzyme requires free formate, provided by PurU (16). If
255 the attenuation caused by deletion of *purT* was related to accumulation of formate, then deletion
256 of *purU* in a Δ*purT* background should eliminate the attenuation.
257
258 Contrary to Δ*purT,* a Δ*purU* mutant showed no growth defect in *E. coli* (16). Interestingly, the
259 opposite was observed in *S.* Typhimurium. The Δ*purT* mutant was not growth arrested, whereas a
260 *purU* deletion in *S.* Typhimurium strain 4/74 resulted in a reduced growth in M9 minimal medium
261 (μ= 0.25±0.002; p-value compared to WT strain <0.05). Complementation by providing the *purU*
262 gene *in trans* did not restore wild type growth; on the contrary, it reduced growth rate further (μ=
263 0.15±0.002) (Table 2). A possible explanation is that provision of PurU *in trans* on a plasmid
264 results in too high enzyme levels and excessive conversion of fTHF into THF and formate. A *purT*
265 deletion in the Δ*purU* mutant restored normal growth (μ= 0.38±0.01). This showed that the slow
266 growth rate of a Δ*purU* mutant was not related to lack of substrate for PurT, and that the growth

attenuation of the Δ*purT* mutant probably was caused by formate accumulation or wasteful conversion of fTHF to THF, which disappears when PurT and PurU are absent in the same bacterium.

In macrophage experiments the Δ*purU* mutant was significantly attenuated in multiplication over 20 hours (4.6 ± 2.5; $p<0.05$). The Δ*purU* mutant was also significantly attenuated in mouse virulence, albeit not to a level that resembled Δ*purN* and Δ*purT* mutants (Table 2). In accordance with the phenotype seen during growth in M9 medium, a completely restored virulence of the Δ*purT*/Δ*purU* double mutant was seen in the mice assay (CI: 1.6) (Table 2).

Glycine but not serine is available for C-1 production during mouse infection

Purine and C-1 metabolism are closely linked, and the reason that PurN and PurT are not functionally redundant *in vivo* during infection of mice could be related to the need to secure sufficient purine synthesis and C-1 metabolism to occur concurrently. Detailed investigations of the role of C-1 metabolism of *S.* Typhimurium in virulence have not been reported, and we therefore undertook a series of characterizations of mutants in the *S.* Typhimurium serine and glycine metabolism.

We have previously predicted and validated by challenge experiments that a Δ*serA* mutant, which is unable to synthesize serine and therefore rely on uptake of serine or production of serine from glycine for serine production (see Fig 1B), is fully virulent in mice (8). Others have likewise predicted by *in silico* modelling that SerA is not needed for growth *in vivo* (5). From this we previously concluded that serine was most likely taken up from the host environment, but that further studies were needed to understand the relative contribution of GlyA and the glycine cleavage enzyme, GVC, in the phenotype of the Δ*serA* mutant (8). In the present study we

analysed a Δ*gcvT* mutation that cannot convert glycine into C-1 units and CO_2 (see Fig 1B). Whether the Δ*gcvT* mutation was present alone or together with the Δ*serA* mutation, virulence was reduced significantly. The strain multiplied like the wild type strain in cultured macrophages, suggesting that the limiting step in infection was not intracellular multiplication in this cell type (Table 3). The results show that exogenous glycine, and not serine as previously believed, was available during infection. If serine had been available in sufficient amounts for synthesis of glycine and C-1 units, then the Δ*gcvT* mutation would have had no effect. This appears to be the situation *in vitro*, since the Δ*gcvT* mutant grew as well as the wild type strain in M9 medium (μ= 0.31±0.21), while the Δ*glyA* mutant was severely growth attenuated. This phenotype could be reversed by addition of the wild type *glyA* gene *in trans,* and addition of glycine to the minimal medium also restored growth partially (Table 3). The wild type 4/74 strain was not affected by addition of glycine to the medium (data not shown). These observations suggest that *in vitro* GlyA is the enzyme that is most important for production of C-1 units, while the glycine cleave system has this role *in vivo*, and it underscores the difficulty in predicting *in vivo* importance from *in vitro* growth phenotypes. We can also conclude from the Δ*gcvT* single mutant, that serine production through the SerA enzyme (present in the Δ*gcvT* single mutant) and subsequent conversion to glycine and C-1 units by the GlyA enzyme (also present) is not sufficient to support virulence of *Salmonella* Typhimurium 4/74 in the mouse model.

These conclusions were corroborated by the analysis of the Δ*glyA* mutant, which was only partly reduced in virulence (Table 3). If glycine were normally synthesized from serine through the GlyA enzyme, then the mutation would have had severe consequences. As expected, the introduction of a Δ*serA* mutation (in itself dispensable) in the Δ*glyA* strain (partly avirulent) resulted in total avirulence, because now the cell had no means of obtaining serine for protein synthesis. For the same reason, Thiele et al. (5) predicted this combination to be lethal in *S.* Typhimurium. Deletion

317 of the *gcvT* gene in the Δ*glyA* background increased attenuation even further than the two single
318 mutations, and no colonies were obtained from mice challenged with this strain. If for statistical
319 purposes one assumed one colony per mice, this would not have been significantly different from
320 the Δ*gcvT* mutant on its own, but significantly more attenuated than the Δ*glyA* mutant (Table 3).
321 The observation was expected, because now the production of C-1 units for purine and methionine
322 synthesis was totally blocked. In macrophages, growth of the Δ*glyA* mutant was highly impaired,
323 and a wild-type *glyA* gene provided *in trans* complemented this phenotype (Table 3), suggesting
324 that either glycine biosynthesis or formation of serine from glycine was a limiting factor for *S.*
325 Typhimurium growth in macrophages, but also that the lower multiplication rate does not lead to
326 total avirulence in mice (CI: 0.3).
327
328 The results with the *glyA* mutant to some extend contradicted a previous report by us (8), where
329 we concluded that the Δ*glyA* deletion mutant was more attenuated in mice than reported here.
330 Upon microscopic analysis of the different mutants we discovered a possible reason for this
331 discrepancy. The Δ*glyA* deletion, and deletion of *glyA* in combination with *gcvT*, resulted in a
332 mixture of normally shaped and elongated bacteria (Supplementary material Fig S2. The altered
333 relation between the optical density and the concentration of colony forming units of the Δ*glyA*
334 mutant cultures may have led us to the overestimation of the importance in virulence in the
335 previous study where we used OD-values to prepare the inoculum, because the elongated cells
336 resulted in a lower mutant-to-wild type ratio in the input pool. In the present study, the infections
337 were performed with a one-to-one ratio based upon number of colony forming units, eliminating
338 this problem. Elongation of cells could be eliminated by complementation *in trans* (data not
339 shown), showing that the cell division was somehow affected by elimination of the GlyA reaction.
340 We did not enquire further into this interesting phenotype in the present study.
341

342 We showed above that virulence attenuation of the Δ*purT* mutant was probably associated to
343 imbalance in formate or fTHF conversion. To investigate whether lack of THF production affected
344 the phenotype of mutants in C-1-metabolism, we characterized a number of combined purine
345 synthesis and C-1 metabolism mutants. All four combinations of Δ*serA* or Δ*glyA* mutations with
346 mutation in *purN* and *purT* were growth attenuated to the same extend as the single Δ*serA* or
347 Δ*glyA* mutants *in vitro* (combinations with Δ*purN*: μ= 0.00 (Δ*serA*) and 0.04 ± 0.003 (Δ*glyA*);
348 combinations with Δ*purT*; μ= 0.00 (Δ*serA*) and 0.03 ± 0.00 (Δ*glyA*)). From this we concluded that
349 there was no significant effect of the extra deletion of *purN* and *purT* in the Δ*serA* and Δ*glyA*
350 mutants *in vitro* even though the competing use of methylene-THF for purine biosynthesis was
351 prevented. We also tested the Δ*purN*/Δ*glyA* strain in the mouse model and this strain was not
352 significantly different from the Δ*purN* mutant on its own (CI: 0.02±0.02), while the Δ*purT*/Δ*serA*
353 mutant apparently was less attenuated than the Δ*purT* strain (CI:0.17±0.13), however the
354 difference was not statistically different from the Δ*purT* strain on its own (p>0.05). Together these
355 experiments indicated little or no overlap between the two systems.
356
357 **Synthesis of methionine from homocysteine and C1-THF *in vivo*.**
358 Methionine synthesis is also interlinked with the purine synthesis through the common pool of
359 mTHF and THF (Fig 1B), and we therefore also considered its importance for the results obtained
360 with *S.* Typhimurium lacking PurN or PurT. Methionine is synthesized by two homocysteine
361 methylating enzymes, MetE and MetH (27). The apparent redundancy of the reaction should
362 enable the strain to synthesize methionine even when either the *metE* or the *metH* gene was
363 mutated, and previous predictions of redundancy in *S.* Typhimurium has predicted that these two
364 enzymes form a cut-set (6). However, while the MetE enzyme is functional under aerobic growth,
365 the MetH enzyme requires vitamin B12, which is only synthesized in *S.* Typhimurium under
366 anaerobic conditions (28). The Δ*metE* mutant was found to be highly growth retarded (μ=

367 0.09±0.03), most probably because the bacterium then relies on the anaerobic enzyme, MetH.
368 Interestingly, however, also a Δ*metH* mutant was found to grow with a lower growth rate than the
369 wild type in M9 medium (μ= 0.20±0.01), suggesting that the MetH enzyme is important under the
370 growth condition tested (Table 4). As expected, a Δ*metE*/Δ*metH* double mutant did not grow in
371 M9 minimal medium, since no methionine is available to this strain for protein synthesis. This
372 phenotype could be complemented by addition of methionine to the medium (μ= 0.31±0.02), a
373 step that did not affect growth of the wild type strain (data not shown) and showing that
374 methionine biosynthesis is dispensable, when methionine can be taken up from the environment.
375 The same was the case for the growth defects of the *ΔmetH* single mutant (μ= 0.35±0.03 with
376 methionine supplied). When the *metE* gene was supplied in trans to the Δ*metE*/Δ*metH* double
377 mutant, the growth was fully restored (μ= 0.24±0.03) to the level of the Δ*metH* single mutant,
378 showing the importance of the MetH enzyme during aerobic growth even with the MetE protein
379 expressed from a multicopy plasmid. Addition of methionine to the Δ*metE* mutant in M9 medium
380 likewise raised the growth rate (μ= 0.20±0.01). A triple mutant, where the double *metE*/*metH*
381 mutations were combined with mutation of *purT* did not grow in M9 media, corresponding to the
382 phenotype of the Δ*metE*/Δ*metH* mutant on its own. Addition of methionine restored growth
383 completely in this mutant (μ= 0.35±0.01), which also corresponded to the Δ*metE*/Δ*metH* mutant
384 on its own. Taken together the results were interpreted as no or very little interaction between
385 PurT and MetH/MetE, and vice versa.
386
387 The Δ*metH* mutant was slightly but significantly attenuated during mouse infection with a
388 competitive index of 0.4 ($p<0.001$), while the Δ*metE* single mutant and the Δ*metE*/Δ*metH* double
389 mutants were totally avirulent (Table 3). Althougt the *metE* gene *in trans* fully complemented the
390 growth phenotype of the double mutant to the level of the Δ*metE* mutant in M9 medium, the
391 presence of the *metE* gene on a multicopy gene did not render the double mutant virulent at all.

392 Methionine appears to be available in macrophages to some extent, as the Δ*metE*/*ΔmetH* double
393 mutant could multiply three-fold within twenty hours in cultured macrophages (Table 4). While
394 methionine is available in macrophages, we may conclude from the infection studies, that it is not
395 available during other phases of mouse infection. Elimination of *purT* in the double mutant
396 background reduced net multiplication rate in macrophages significantly (0.73±0.29), suggesting a
397 synergistic effect of the mutations at this level.
398

399 ## Discussion

400 The main aim of this study was to determine the contribution of the *in vivo* redundant enzymes
401 PurN and PurT to virulence in *S.* Typhimurium, and when realizing that they were not redundant *in*
402 *vivo* during infection of mice, to elucidate possible reasons for this. In the broader perspective, the
403 study illustrates that one cannot safely assume that *in vitro* redundancy between enzymes is
404 followed by a similar *in vivo* redundancy during infection of mice. Another important observation,
405 based on the *in vitro* growth experiments, is that even though *E. coli* and *S.* Typhimurium share
406 the basic architecture of the metabolic systems we have investigated, the growth phenotypes
407 associated with knock out of a gene cannot always be assumed to be the same. This notion has
408 recently been highlighted in another publication, dealing with thiamine biosynthesis in *E. coli* (29).
409

410 The concept of redundancy has mainly been understood from studies of *E. coli* K12. When it grows
411 on glucose as the sole carbon source, more than 80 of the 227 metabolic enzymes are non-
412 essential (30). Redundancy may be a trade-off between efficiency and robustness, and organisms
413 with a broad niche repertoire show the highest degree of redundancy. Therefore, redundancy has
414 been interpreted as a mechanism that supports niche adaptation (31). Others, however, consider
415 redundancy as a way to withstand detrimental mutations (32). Our observations with PurN and
416 PurT, and MetE and MetH for that matter, suggest that some pairs of redundant enzymes may be

417 artefacts of studying bacteria in test tubes, and that the two enzymes are maintained because
418 they are both essential, possibly at different steps in the normal live cycle (infection steps in the
419 case of pathogenic bacteria). This corresponds mostly to the niche adaptation theory, since the
420 likely explanation for both enzymes being essential is that *Salmonella* goes through a series of
421 different environments (niches) in the infection process. However, in the niche adaptation theory,
422 the need for both of the enzymes is not absolute.
423
424 The total avirulence of both PurN and PurT mutants could be complemented by addition of the
425 respective enzyme encoded from a plasmid *in trans*. This proved that the attenuation was related
426 to the lack of the specific enzymes. It is well known that purine biosynthesis is required for
427 intracellular multiplication, and purine biosynthesis mutants have been employed as live vaccines
428 against *S.* Typhi (33) and *S.* Typhimurium (34), as well as *Mycobacterium tuberculosis* (35),
429 *Brucella melitensis* (36) and *Franscisella tularensis* (37). In light of this it was not surprising that
430 the double Δ*purN*/Δ*purT* mutant was attenuated and unable to multiply inside macrophages.
431 However, previous studies on putative targets genes for attenuated life vaccines have ignored the
432 3rd step in the biosynthesis of purines, catalysed by PurN and PurT, due to the recognized
433 redundancy between the enzymes *in vitro.* Based on our results, deletion mutants in either PurN or
434 PurT are likely also to be good vaccine candidates against *Salmonella*.
435
436 Studies of multiplication ability in cultured macrophages is a sensitive method to identify virulence
437 attenuation in *Salmonella*, and Leung and Finlay (38) showed that mutants that could not multiply
438 in cultured macrophages were also avirulent in mice. The opposite, however, is not necessarily the
439 case. The avirulent PurT mutant was not critically affected in propagation in the intracellular
440 environment of J774A.1 macrophages. This suggested that the interaction with this cell type was
441 not the limiting point for the PurT mutant. Contrary to the Δ*purT* mutant, the Δ*purN* mutant grew

poorly or was killed more quickly than wild type inside macrophages (these two things cannot be separated by the assay we used), suggesting that macrophage survival/growth could be one of the critical points in progression in the host for this mutant. Recent studies have shown that one should be careful not to over-interpret results from experiment with cultured macrophages, and especially not to draw conclusions on mechanism of virulence from such studies (39). Also it should be noted that the J774 cells have a different genetic background (BalB/c) than the mouse strain used, and even though they are both *slr*$^{-/-}$ (N-ramp^{neg}), this may make it difficult to compare the *in vivo* and the *in vitro* situation directly, Our results with macrophages should thus be interpreted with caution.

Studies using a mutant in the essential *S.* Typhimurium *purG* gene (also termed *purL*) have shown that this purine auxotroph strain fail to repair DNA damage caused by Reactive Oxygen Species in the phagosome environment of the macrophage, and that this can explain the inability of the mutant to multiply inside macrophages (40). Our results suggest that PurN has sufficient activity within macrophages to ensure that DNA damage can be repaired in the absence of PurT, while PurT cannot ensure wild type propagation in macrophages in the absence of PurN. The reduction in net growth of the PurN mutant with approximately 50 % corresponds to previous observations on net growth rate of a Δ*purH* mutant in mouse spleens (26). The results indicated that intra cellular propagation was the rate-limiting step causing attenuation of the *purN* mutant, and also that purines cannot be supplied in sufficient amounts from external sources during macrophage infection. A recent study has shown that the Δ*purH* mutant grows with two distinct populations with respect to location and growth rate in the spleen of mice (26), probably reflecting that the growth happens in two compartments with two different demands for purine synthesis, and it would be interesting to investigate whether the Δ*purN* mutant also grows as two separate populations inside macrophages.

467

468 Purine synthesis has been found to be marginally (down) regulated when *S.* Typhimurium grows
469 inside cultured macrophages, and PurN and PurT are regulated to the same degree, although only
470 PurN was significant different from the control by the statistical analysis used in the study (41).
471 The reference condition was growth of opsonized bacteria in cell culture media, and the
472 observation is therefore difficult to compare to the present study, where bacteria were grown in LB
473 media prior to macrophage challenge; however, it indicates that *S.* Typhimurium requires as much
474 or slightly more purine *de novo* biosynthesis to grow in cell culture medium as it requires for
475 growing inside macrophages.

476

477 With current techniques it is not possible to determine the exact amounts of important
478 intermediates in the purine biosynthesis, such as THF species, in bacteria during infection. In order
479 to understand why both enzymes were essential and whether this was related to imbalance on the
480 consumption and production of THF species, we chose a mutant-based approach, where relevant
481 genes were deleted in different combinations. Our studies with Δ*purU* and Δ*purT* strains strongly
482 suggested that a main reason for attenuation of the *purT* mutant was accumulation of formate or
483 wasteful use of fTHF by PurU, since the double Δ*purT*/Δ*purU* mutant was fully virulent. For this to
484 make sense, the metabolism should be aerobic, since this is the condition where formate
485 production by PurU is a dead end product in the absence of PurT (16). In this sense, our
486 observation supports a recent study, showing that the environment perceived by *Salmonella*, when
487 it resided in cells of the monocyte line in the spleen, is aerobic, because *Salmonella* exclusively
488 resides in the red pulp in close proximity to erythrocytes (26). It still needs to be determined
489 whether formate accumulation or wasteful fTHF, or both, are the important factor, and to detect
490 the exact point in infection, where this may be the case. The fact that the mutant without PurT

491 could propagate in cultured macrophages suggested that formate accumulation and/or wasteful
492 fTHF consumption is not a problem inside such cells.
493
494 The current study also made important observations with regard to the role of C-1-metabolism in
495 virulence. Based on *in vitro* experiments, the glycine cleavage enzyme, GCV, has been estimated
496 to contribute less to formation of C-1 units in *E. coli* than the hydroxyl-methyltransferease-enzyme
497 encoded by *glyA* (14). The growth experiments in the current study shows that *S.* Typhimurium *in*
498 *vitro* shows the same balance between the two enzymes, since the Δ*gcvT* mutant was not growth
499 attenuated, while the Δ*glyA* mutant did not grow. *In vivo,* however, the situation was totally
500 opposite. The Δ*glyA* strain was only partly attenuated, while the Δ*gcvT* mutant was highly
501 attenuated and colonies were rarely isolated from any infected animals. Interestingly, concurrent
502 mutation in *serA* did not change this, which showed that contrary to previously expected (8),
503 exogenous glycine, and not serine must be available to *Salmonella* during infection. Else, glycine
504 and C-1 units could have been formed by *glyA* from serine, and then the Δ*gcvT* mutation would
505 have had no effect (Figure 1B). When Δ*purN* or Δ*purT* mutations were combined with mutation of
506 *serA* or *glyA*, the *in vitro* phenotypes indicated that the purine synthesis and C-1 metabolism
507 systems did not interfere significantly with each other. Double mutants caused the same
508 phenotype as the single gene with the highest influence on the performance of *S.* Typhimurium.
509 Unexpectedly we discovered that Δ*glyA* mutants form elongated cells when growing *in vitro*.
510 Filament formation is well known in *Salmonella* as a response to osmotic and cold stress (42,43),
511 but this phenotype of *glyA* mutation has, to the best of the author's knowledge, not previously
512 been described in this genus, nor in any of its close relatives. The phenotype could be reversed by
513 genetic complementation *in trans*, ruling out that it was caused by secondary mutations in genes
514 of relevance for Z-ring formation.
515

In the process of detailing the link between the purine and methionine metabolism we discovered that the enzymes MetE/MetH, which are considered redundant (6) also did not show redundancy during infection. We confirmed that methionine is not taken up from the host during mice infection, since the Δ*metE*/Δ*metH* double mutant was avirulent. It has previously been shown that mutation of *metC*, which mobilises sulphur for methionine, is essential in *S.* Typhimurium (44), and *S.* Typhimurium is only one among several pathogens where methionine biosynthesis appears to be essential during growth in the animal host (45,46). *In vitro,* the double mutant did not grow either. This phenotype could be complemented by addition of methionine to the medium, showing that the methionine uptake system can compensate fully for lack of the biosynthesis system during growth *in vitro*. Provision of *metE in trans* restored growth to the level of the *ΔmetH* mutant. This demonstrated that the *metE* mutation can be complemented *in trans* and that MetE and MetH are not fully redundant; the enzyme MetH, which is associated to anaerobic growth (26), must play a role during this growth, even though the cultures were shaken. Further studies are needed to fully understand the reasons for this observation. Like for the C-1 metabolism, we did not find any indication that purine mutation significantly affected the phenotypes obtained after mutation of methionine synthesis genes.

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679 **Table 1. Strains and plasmids used in the study.**
680

Strain number	Strain	Genotype and marker[#]	Source
Jeo3774	*S.* Typhimurium 4/74	Wild- type	(47)
Jeo1473	*S.* Typhimurium Δ*purN*::Cm	4/47 ΔSTM474_2603, cm^r	This study
Jeo1516	*S.* Typhimurium Δ*purN*::Cm-comp.	4/47 ΔSTM474_2603+ STM474_2603^{comp}, cm^r, amp^r	This study
Jeo1496	*S.* Typhimurium Δ*purT*::Kan	4/47 ΔSTM474_1915, kan^r	This study
Jeo1517	*S.* Typhimurium Δ*purT*::Kan-comp.	4/47 ΔSTM474_1915+ STM474_1915^{comp}, kan^r, amp^r	This study
Jeo1509	*S.* Typhimurium Δ*purN*::Cm/ Δ*purT*::Kan	4/74 ΔSTM474_2603/ ΔSTM474_1915, kan^r, cm^r	This study
Jeo1512	*S.* Typhimurium Δ*glyA*::Kan	4/47 ΔSTM474_2659, kan^r	(8)
Jeo1528	*S.* Typhimurium	4/47	This study

	Δ*glyA*::Cm	ΔSTM474_2659, cm^r	
Jeo1570	*S.* Typhimurium Δ*glyA*::Kan- comp.	4/47 ΔSTM474_2659 + STM474_2659^{comp}, kan^r, amp^r	This study
Jeo1526	*S.* Typhimurium Δ*serA*::Kan	4/47 ΔSTM474_3209, kan^r	This study
Jeo1514	*S.* Typhimurium Δ*serA*::Kan/ Δ*glyA*::Cm	4/47 ΔSTM474_3209 + ΔSTM474_2659 kan^{r,} cm^r	This study
Jeo1527	*S.* Typhimurium Δ*purT*::kan/ Δ*glyA*::cm	4/74 ΔSTM474_1915/ ΔSTM474_2659, kan^r, cm^r	This study
Jeo1522	*S.* Typhimurium Δ*purT*::kan/ Δ*serA*::cam	4/74 ΔSTM474_1915/ ΔSTM474_3209, kan^r, cm^r	This study
Jeo1521	*S.* Typhimurium Δ*purN*::Kan/ Δ*glyA*::Cm	4/74 ΔSTM474_2603/ ΔSTM474_2659, kan^r, cm^r	This study
Jeo1525	*S.* Typhimurium Δ*purN*::Kan/ Δ*serA*::Cm	4/74 ΔSTM474_2603/ ΔSTM474_3209,	This study

		kan^r, cm^r	
Jeo1523	*S.* Typhimurium Δ*purU*::Kan	4/47 ΔSTM474_1773, kan^r	This study
Jeo1577	*S.* Typhimurium Δ*purU* -compl	4/47 ΔSTM474_1773+ ΔSTM474_1773^{compl}, amp^r	This study
Jeo1572	*S.* Typhimurium Δ*purU* /Δ*purT*::kan	4/47 ΔSTM474_1773, ΔSTM474_1915, kan^r	This study
Jeo1529	*S.* Typhimurium Δ*gcvT*::Cm	4/47 ΔSTM474_3202, cm^r	This study
Jeo1531	*S.* Typhimurium Δ*serA*::Kan/ Δ*gcvT*::Cm	4/74 ΔSTM474_3209/ ΔSTM474_3202, kan^r, cm^r	This study
Jeo1530	*S.* Typhimurium Δ*glyA*::Kan/ Δ*gcvT*::Cm	4/74 ΔSTM474_2659/ ΔSTM474_3202, kan^r, cm^r	This study
Jeo1574	*S.* Typhimurium Δ*metE*::Kan	4/47 ΔSTM474_4143, kan^r	This study

Jeo1590	*S.* Typhimurium Δ*metH*::Cm	4/47 ΔSTM474_4378, Cm^r	This study
Jeo1593	*S.* Typhimurium Δ*metE*::Kan/ Δ*metH*::Cm	4/74 ΔSTM474_4143/ ΔSTM474_4378, kan^r, cm^r	This study
Jeo1599	*S.* Typhimurium Δ*metE*::Kan/ Δ*metH*::Cm-comp.	4/74 ΔSTM474_4143/ ΔSTM474_4378/+ $STM474\_4143^{comp}$	This study
Jeo1594	*S.* Typhimurium Δ*metE*::Kan/ Δ*metH*::Cm, Δ*purT*::Kan	4/74 ΔSTM474_4143/ ΔSTM474_4378/ ΔSTM474_1915, kan^r, cm^r	This study
-	*S.* Typhimurium Δ*ssaV*::Kan	4/47 ΔSTM474_1420, kan^r	(21)
-	pKD3	Template plasmid for amplification of chloramphenicol resistance gene cassette. Amp^r, Cm^r	(20)
-	pKD4	Template plasmid for amplification of	(20)

		kanamycin resistance gene cassette. Amp^r, Kan^r	
-	pKD46	Lambda red mediated recombination system. Amp^r	(20)
-	pACYC177	Low copy number plasmid used for complementation. Amp^r.	(48)

*Gene numbers refer to the 4/74 genome annotation in BioCyC (www.biocyc.org). Cm^r: chloramphenicol resistant through insertion of the chloramphenicol gene cassette from the plasmid pKD3. Kan^r: Kanamycin resistant through the insertion of the kanamycin resistance gene cassette from the plasmid pKD4. Comp: Complemented *in trans* by cloning of the deleted gene on the plasmid pACY177.

687 **Table 2. Growth and Virulence of S. Typhimurium 4/74 pur-mutants.**

Strain	Mutant	Growth rate[a,b] (μ, hours^{-1}))	Mice virulence (2)[b]	J774A.1 macrophage[c] (multiplication/20 h)[b]
Jeo3774	WT 4/74	0.37±0.017	1.00	9.7 ± 2.5
Jeo1473	Δ*purN*	0.35±0.01	0.03 ± 0.0***	4.4 ± 3.5*
Jeo1516	Δ*purN* / pACYC177*purN*	0.31±0.08	0.60 ± 0.2	ND
Jeo1496	Δ*purT*	0.36±0.02	0.02 0.01***	8.0 ± 5.6
Jeo1517	Δ*purT* / pACYC177*purT*	0.37±0.01	0.90 ± 0.3	ND
Jeo1509	Δ*purN* / Δ*purT*	NG	0.00 ± 0.0^{NT}	0.3 ± 0.2****
Jeo1523	Δ*purU*	0.25±0.002***	0.60 ± 0.8	4.6 ± 2.5*
Jeo1575	Δ*purU* / pACYC177*purU*	0.15±0.002***	ND	2.1 ± 0.1*
Jeo1572	Δ*purU* / Δ*purT*	0.38±0.01	1.63 ± 0.6**	ND

688 ND: not determined; NG: no growth in M9 + glucose medium.

689 a: Specific growth rate *in vitro* in M9+glucose (ln(2)/doubling time).

690 b: *: p<0.05; **: P-value <0.01 ***: p-value <0.001; ****: p<0.0001; NT: Cannot be tested as

691 no mutant colonies were recovered from challenged mice.

692 c: Control 4/74 Δ*ssaV:* 2.1 ± 0.6***

693

694 **Table 3. Growth and virulence phenotypes of mutants in glycine and serine metabolism**

695 **in *S.* Typhimurium 4/74.**

Strain	Genotype	Growth rate[a,b] (μ, hours^{-1})	Mice virulence (2)	Macrophage multiplication (1h to 21 h)[c]
Jeo3774	Wildtype (WT) 4/74	0.37±0.017	1.00	6.9 ± 3.4
Jeo1529	Δ*gcvT*	0.36±0.002	0.03 ± 0.03^{***e}	7.2 ± 3.9
Jeo1531	Δ*serA* Δ*gcvT*	ND	0.05 ± 0.04***	ND
Jeo1522	Δ*serA* Δ*purT*	NG	0.15 ± 0.15***	ND
Jeo1512	Δ*glyA*	0.03±0.003	0.3 ± 0.4*	0.3 ± 0.03**
Jeo1512	Δ*glyA*+gly[d]	0.20±0.01	NR	NR
Jeo1570	Δ*glyA*+pACYC177*glyA*	0.35±0.01	ND	5.6 ± 1.50[e]
Jeo1514	Δ*glyA* Δ*serA*	NG	0.01 ± 0.01***	ND
Jeo1530	Δ*glyA* Δ*gcvT*	NG	0.0 ± 0.0$^{NT(*)}$	ND
Jeo1521	Δ*glyA* Δ*purN*	0.03±0.003	0.02 ± 0.02***	1.2 ± 1.0***

696 ND: not done; NG: no growth in M9 + glucose medium.

697 a: Specific growth rate *in vitro* in M9+glucose (ln(2)/doubling time).

698 b: *: p<0.05; **: P-value <0.01 ***: p-value <0.001; ****: p<0.0001;

699 NT: This strain could not be tested statisically as no mutant colonies were recovered from

700 challenged mice. If for statistical purposes, one assumed one colony to be obtained from each

701 mouse of this double mutant, the results would not have been significantly different from the

702 competitive index of the Δ*gcvT* mutant on its own (p=0.06) but significantly different from the

703 *glyA* mutant (p=0.03).

704 c: Control 4/74 Δ*ssaV:* 2.1 ± 0.6***

705 d: Growth in M9 minimal medium with supplement of glycine (gly).

706 e: Multiplication rate is significantly different from Jeo1512 (Δ*glyA*) and not different from Jeo3774

707 (wild type strain).

708

709 **Table 4. Virulence phenotypes of *metE* and *metH* mutants in *S.* Typhimurium 4/74.**

Strain number	Genotype	Growth rate[a,b] (μ) gene/h	Competitive index (2)	Multiplication rate in macrophages (1h to 21 h)[d]
Jeo3774	Wildtype (WT) 4/74[c]	0.37±0.02	1.00	6.9 ± 3.4
Jeo1590	Δ*metH*	0.20±0.01	0.4 ± 0.2	ND
Jeo1590	Δ*metH*+met[c]	0.33±0.03	NR	NR
Jeo1574	Δ*metE*	0.06±0.01	0.0 ± 0.0	ND
Jeo1574	Δ*metE*+met[c]	0.23±0.05	NR	NR
Jeo1593	Δ*metH* Δ*metE*	NG	0.0 ± 0.0[NT]	2.9 ± 0,4
Jeo1593	Δ*metH* Δ*metE*+met[c]	0.26±0.005	NR	NR
Jeo1599	Δ*metH* Δ*metE* /pAYCY177*metE*	0.24±0.002	0.00 ± 0.0[NT]	ND

710 ND: not done; NG: no growth in M9 + glucose medium.

711 a: growth rate *in vitro* in M9+glucose.

712 b: *: p<0.05; **: P-value <0.01 ***: p-value <0.001; ****: p<0.0001; NT: Cannot be tested as

713 no mutant colonies were recovered from challenged mice.

714 c: Growth in M9 minimal medium with supplement of methionine

715 d: Control 4/74 Δ*ssaV:* 2.1 ± 0.6***

716

Fig 1. The third reaction-step in the purine *de novo* synthesis carried out by the *in vitro* redundant enzymes PurN and PurT (Fig. 1A), and the interconnection between purine synthesis and the carbon-1 metabolism, where essential carbon-1 (C-1) units are produced (Fig 1B).
(Fig 1A): PurN converts Gar to fGar using fTHF as formyl donor, while PurT converts Gar to fGar using formate as formyl donor. Formate is produced from fTHF by the enzyme PurU. (B): The production of C-1 units for amino acid and purine synthesis in *S.* Typhimurium happens when GlyA converts serine into glycine and when glycine is converted into ammonia and carbon dioxide by the glycine cleavage system. The pools of formyl-THF (fTHF), THF and methylene-THF (mTHF) are shared between the purine and the Carbon-1 synthesis pathways.

Fig. 1A

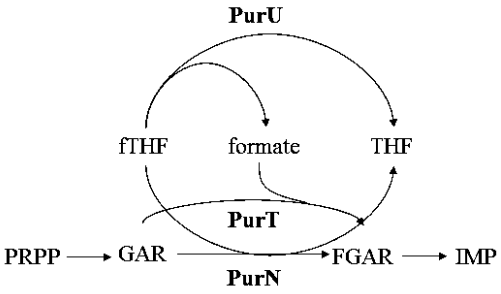

Fig. 1B