INTRODUCTION

*Helicobacter pylori* (H. pylori) was first described in 1983 and since then it has been a subject of intense research (Warren & Marshall, 1983). The human pathogen colonizes the stomach of about 50% of the world’s population (Hooi et al., 2017). While most infected individuals remain asymptomatic during their lifetime, *H. pylori* is considered to be the main cause of gastric cancer, which accounted for about 810,000 deaths worldwide in 2018 (de Martel et al., 2020). Additionally, the pathogen is responsible for a number of other diseases e.g. peptic ulcer disease, which is declining in prevalence, although to a regionally different extent (Lanas & Chan, 2017).

Substrate usage determines carbon flux via the citrate cycle in *Helicobacter pylori*

Thomas M. Steiner1 | Clara Lettl2,3 | Franziska Schindele2 | Werner Goebel2 | Rainer Haas2,3 | Wolfgang Fischer2,3 | Wolfgang Eisenreich1

1Bavarian NMR Center—Structural Membrane Biochemistry, Department of Chemistry, Technische Universität München, Garching, Germany
2Chair of Medical Microbiology and Hospital Epidemiology, Max von Pettenkofer Institute of Hygiene and Medical Microbiology, Faculty of Medicine, LMU Munich, München, Germany
3German Center for Infection Research (DZIF), Partner Site Munich, München, Germany

Correspondence
Wolfgang Fischer, Chair of Medical Microbiology and Hospital Epidemiology, Max von Pettenkofer Institute of Hygiene and Medical Microbiology, Faculty of Medicine, LMU Munich, München, Germany.
Email: Fischer@mvp.lmu.de
Wolfgang Eisenreich, Bavarian NMR Center—Structural Membrane Biochemistry, Department of Chemistry, Technische Universität München, Garching, Germany.
Email: wolfgang.eisenreich@mytum.de

Funding information
German Center for Infection Research (DZIF), Grant/Award Number: 8025806810; Deutsche Forschungsgemeinschaft, Grant/Award Number: 364653263

Abstract

*Helicobacter pylori* displays a worldwide infection rate of about 50%. The Gram-negative bacterium is the main reason for gastric cancer and other severe diseases. Despite considerable knowledge about the metabolic inventory of *H. pylori*, carbon fluxes through the citrate cycle (TCA cycle) remained enigmatic. In this study, different 13C-labeled substrates were supplied as carbon sources to *H. pylori* during microaerophilic growth in a complex medium. After growth, 13C-excess and 13C-distribution were determined in multiple metabolites using GC–MS analysis. [U-13C6]Glucose was efficiently converted into glyceraldehyde but only less into TCA cycle-related metabolites. In contrast, [U-13C5]glutamate, [U-13C4]succinate, and [U-13C4]aspartate were incorporated at high levels into intermediates of the TCA cycle. The comparative analysis of the 13C-distributions indicated an adaptive TCA cycle fully operating in the closed oxidative direction with rapid equilibrium fluxes between oxaloacetate—succinate and α-ketoglutarate—citrate. 13C-Profiles of the four-carbon intermediates in the TCA cycle, especially of malate, together with the observation of an isocitrate lyase activity by in vitro assays, suggested carbon fluxes via a glyoxylate bypass. In conjunction with the lack of enzymes for anaplerotic CO2 fixation, the glyoxylate bypass could be relevant to fill up the TCA cycle with carbon atoms derived from acetyl-CoA.

KEYWORDS

*Helicobacter pylori*, isocitrate lyase, isotopologue profiling, metabolic adaption, metabolism, TCA cycle

1 | INTRODUCTION

*Helicobacter pylori* (H. pylori) was first described in 1983 and since then it has been a subject of intense research (Warren & Marshall, 1983). The human pathogen colonizes the stomach of about 50% of the world’s population (Hooi et al., 2017). While most infected individuals remain asymptomatic during their lifetime, *H. pylori* is considered to be the main cause of gastric cancer, which accounted for about 810,000 deaths worldwide in 2018 (de Martel et al., 2020). Additionally, the pathogen is responsible for a number of other diseases e.g. peptic ulcer disease, which is declining in prevalence, although to a regionally different extent (Lanas & Chan, 2017).
Even though there is an established treatment of either first-line triple-therapy or second-line quadruple-therapy (Malfertheiner et al., 2017), the efficiency of the first-line therapy has declined below 80% in many countries (Fock et al., 2009; Megraud et al., 2013). There are still alternatives and additives for the current treatment, e.g. susceptibility-guided treatment, bismuth-therapy, or the usage of the potassium-competitive acid blocker vonoprazan as an alternative to proton pump inhibitors (O’Connor et al., 2019). Nevertheless, new means to fight resistant *H. pylori* are needed. The central bacterial metabolism is a promising target for novel antibiotics (Bhargava & Collins, 2015; Bishai, 2017; Murima et al., 2014). However, as a prerequisite for metabolism-guided drug development, full knowledge about the functional metabolic pathways of bacterial pathogens is required (Bhargava & Collins, 2015). To this aim, the unbiased, observation driven analysis of bacterial metabolism is timely as many species differ from the established metabolic pathways determined for model species. There are even metabolic differences within a species that can allow for resistance (Zampieri et al., 2017). Moreover, genome sequences alone hardly provide a complete view upon functional metabolism, especially under medically relevant conditions.

*H. pylori* is known to metabolize different carbon sources, especially glucose and amino acids. Metabolic usage of alanine, proline, arginine, aspartate, glutamate and serine was shown during growth in continuous culture (Stark et al., 1997). Arginine, histidine, isoleucine, leucine, methionine, phenylalanine and valine were shown to be essential for *H. pylori* (Reynolds et al., 1994). Glucose utilization was demonstrated (Hazell et al., 1997) either via the Entner-Doudoroff-pathway (ED-pathway) or the pentose phosphate pathway (PPP) (Mendz et al., 1994a). These pathways depend on a species-specific glucokinase in difference to the more general hexokinase found in many other organisms (Mendz et al., 1993a). NMR analysis of *H. pylori* cell extracts grown in medium supplemented with [U-13C6]glucose identified 13C-labeled lactate as the main product, suggesting an active lactate fermentation pathway (Mendz et al., 1993b). Subsequently, experiments with 13C-labeled pyruvate yielded labeled lactate, acetate, formate, and succinate, which were secreted into the medium. This metabolism is generally referred to as mixed acid fermentation, wherein pyruvate is metabolized to different organic acids without participation of oxygen. On this basis, it can be assumed that reduction of pyruvate into lactate serves to regenerate NAD+, while transformation of pyruvate to acetyl-phosphate and further to acetate produces ATP (Mendz et al., 1994b). Formate is most likely produced through the pyruvate formate lyase reaction (Mendz et al., 1994b). Additionally, secretion of succinate suggests active fumarate reduction to regenerate reducing equivalents under microaerophilic conditions (also cf. Figure 1). Pyruvate was reported to enter the TCA cycle either by anaplerotic reactions, i.e., by carboxylation to oxaloacetate or malate, or via oxidation to acetyl-CoA, although genes encoding a potential pyruvate dehydrogenase complex are missing in the genome of *H. pylori* (Inamoto et al., 1995). H13CO3− was used as a tracer to detect potential carboxylation reactions in *H. pylori* (Hughes et al., 1995; St Maurice et al., 2007). These experiments excluded anaplerotic activities in general, such as reactions catalyzed by pyruvate carboxylase or PEP carboxykinase. However, the data indicated action of a novel four-subunit pyruvate flavodoxin oxidoreductase (PFOR, enzyme 1 in Figure 1) and a pyruvate formate lyase converting pyruvate to a mixture of acetyl-CoA and formate in a reversible reaction (Hughes et al., 1998). These enzymes replace pyruvate dehydrogenase, which is typically observed in organisms operating an oxidative metabolism.

The architecture of the TCA cycle in *H. pylori* is still puzzling. There was experimental evidence for a closed and fully functioning TCA cycle (Ge, 2002; Hoffman et al., 1996). Notably, however, no activity for succinate dehydrogenase could be detected in the earlier studies, leaving the cycle incomplete (Pitson et al., 1999). In an alternative scenario, an incomplete, bifurcate topology of the TCA cycle was proposed (Han et al., 2018; Lee et al., 2017; Pitson et al., 1999) in which the cycle is split into two branches. Here, the putative oxidative branch produces succinyl-CoA from citrate via 2-oxoglutarate, while the reductive branch forms succinate from oxaloacetate through malate and fumarate. Indeed, carbon fluxes via an active succinate dehydrogenase or a reversibly acting fumarate reductase could be shown (Chen et al., 1999; Ge, 2002; Kather et al., 2000).

Similar experimental ambiguity was reported for a potential glyoxylate shunt where the TCA cycle intermediate isocitrate is transformed into succinate and glyoxylate. The latter product could then assemble with acetyl-CoA to form malate. Indeed, activity for malate synthase converting acetyl-CoA and glyoxylate into malate was detected on the basis of NMR assays, but there were no indications for an active isocitrate lyase (Pitson et al., 1999). On the other hand, earlier photometric assays suggested an active isocitrate lyase (Hoffman et al., 1996), leaving the question about a glyoxylate bypass in *H. pylori* unanswered.

Analysis of the *H. pylori* genome using the COG and KEGG databases (Galperin et al., 2015; Kanehisa et al., 2016) as well as recent annotations (Karlsson et al., 2016; Resende et al., 2013), suggests a fully functional TCA cycle with some alterations to the canonical enzymes (enzymes 2, 3, and 4 in Figure 1). Activity of these alternative enzymes has been observed experimentally. The reaction from 2-oxoglutarate to succinyl-CoA (enzyme 2) uses ferredoxin as an oxidizing agent instead of NAD+ (Hughes et al., 1998); succinyl-CoA is transformed to succinate through a CoA-transferase (enzyme 3) using acetooacetate as the CoA-acceptor (Corthes-Thuolaz et al., 1997). Finally, malate oxidation to oxaloacetate uses quinone as the electron-acceptor (enzyme 4) (Kather et al., 2000). Although no designated succinate dehydrogenase is found in the genome, this reaction could be carried out by the respective fumarate reductase, whose bidirectionality was already observed, although with a strong preference for fumarate reduction (Lancaster et al., 2002). It is also noteworthy that genes encoding malate synthase or isocitrate lyase have not been annotated in the genome of *H. pylori*.

To investigate the carbon fluxes in the overall central metabolism of bacteria, labeling experiments starting from stable 13C-enriched substrates (such as glucose, acetate, amino acids) and in-depth isotopologue profiling of the resulting metabolic products are now
proven technologies (Buescher et al., 2015; Eisenreich et al., 2006; Long & Antoniewicz, 2019). In a typical setting, bacteria are grown in the presence of the labeled precursors for an appropriate period. After that, metabolic products are isolated from the cells and analyzed by mass spectrometry and/or NMR spectroscopy. Based on these analytical data, the $^{13}$C-enrichments and their positional distribution can be determined in considerable detail. For example, the relative fractions of specific isotopologues (molecular species containing a given number of $^{13}$C-atoms) assign the efficacies and pathways of substrate usages in the functional metabolic networks under study. As examples, this approach helped to elucidate metabolic pathways and fluxes in a variety of bacterial pathogens, including *Campylobacter jejuni*, a close relative to *H. pylori* (Gao et al., 2017).

Especially, $^{13}$C-labeling experiments with intracellular pathogens served to elucidate carbon usage and fluxes in bipartite metabolic networks (Grubmüller et al., 2014; Häuslein et al., 2016). In these bipartite topologies, two or more carbon substrates are utilized, with each substrate fulfilling a distinct metabolic role. In a simplified view, one class of substrates is used to produce sugars for cell wall synthesis and another class of substrates serves to produce energy. This co-utilization of multiple substrates allows for more metabolic flexibility during changes in the nutritional environment throughout infection and additionally reduces the metabolic burden on the host, as no selective deprivation of a single substrate occurs due to infection (Best & Abu Kwaik, 2019; Eisenreich et al., 2015, 2017, 2019).

We now have exploited the $^{13}$C-technology for a comprehensive

---

**FIGURE 1** $^{13}$C-Profiles in a model for the central carbon metabolism of *H. pylori* starting from [U-$^{13}$C$_6$]glucose. Red arrows indicate $^{13}$C-incorporation via direct transformation of the substrate. Green arrows indicate carbon flux following the decarboxylation of oxaloacetate to pyruvate. Purple arrows indicate flux via the PPP. Colored circles signify the theoretical labeling positions for the respective isotopologue, while orange circles show isotopologues arising through propagation of labeled molecules through the TCA cycle. Percentages represent the observed $^{13}$C-excess for the respective isotopologue. Metabolites in boxes were directly analysed via GC–MS. Numbers in boxes indicate unusual enzymes in the metabolism of *H. pylori*: 1: pyruvate-ferredoxin oxidoreductase; 2: 2-oxoglutarate-ferredoxin oxidoreductase; 3: succinyl-CoA:acetoacetate-CoA transferase; 4: malate:quinone oxidoreductase; 5: (4S)-4-hydroxy-2-oxoglutarate aldolase. PPP: pentose phosphate pathway; KDPG: 2-keto-3-deoxy-phosphogluconate; GAP: glyceraldehyde-3-phosphate; PEP: phosphoenolpyruvate; MTHF: methylytetrahydrofolate; Acetyl-P: acetyl-phosphate; MQ: menaquinone; Fd: ferredoxin.
analysis of substrate usages in the core carbon metabolism of *H. pylori* with a special focus on carbon fluxes via the TCA cycle.

2 | RESULTS AND DISCUSSION

2.1 | Glucose and amino acids serve as main carbon substrates for growth of *H. pylori* in *Brucella* broth medium

To assess the metabolic capabilities of *H. pylori*, we grew the pathogen in the complex *Brucella* broth medium containing 10% fetal calf serum (BB/FCS). Growth in minimal medium could provoke stress response and lead to a degenerate metabolic phenotype, which is not representative for the actual metabolic capabilities of the pathogen. Additionally, *H. pylori* resides in the mucus layer in close proximity to human gastric epithelial cells (Dunne et al., 2014; Schreiber et al., 2004). In this environment, various carbon substrates provided by the epithelial cells most probably serve as nutrients for bacterial survival and replication during infection (Hirayama et al., 2009; Tan et al., 2009). In conclusion, an in vitro setting where the bacteria are grown in the presence of several potential carbon sources appeared superior to a setting where the bacteria are replicating under precarious conditions such as in a minimal medium with a limited supply of potential carbon substrates. Moreover, there are no reports about carbon catabolite repression being active in *H. pylori* (Windham et al., 2018) again suggesting the parallel usage of multiple carbon substrates, as already observed for other pathogens (Eisenreich et al., 2017).

To assess the amounts of potential substrates in BB/FCS, especially of those compounds, which were added as $^{13}$C-tracers in the labeling experiments described below, we analyzed the dried medium by GC–MS (Table 1). To account for protein/peptide-bound amino acids, the medium was also hydrolyzed using hydrochloric acid and the resulting hydrolysate was again analyzed by GC–MS. Free amino acids were found at amounts of 0.4–4.1 mM only. Notably, the concentrations of those amino acids, which were supplied as $^{13}$C-tracers in the labeling experiments, did not significantly exceed 1 mM. As expected, these levels increased to 2.8–20.1 mM when the proteins/peptides in the medium were hydrolyzed under harsh acidic conditions. Glycerol was present at a low concentration of 0.5 mM, while succinate was found at minor amounts of 0.1 mM. Citrate as well as acetate were virtually absent (<0.01 mM). According to the manufacturer’s specifications, glucose was present at a concentration of 4.5 mM in BB/FCS medium. On this basis, glucose and peptides/amino acids can be considered as the main carbon substrates for *H. pylori* grown in BB/FCS medium. In the labeling experiments, $^{12}$C-tracers were added at concentrations of 5 or 10 mM. Thus, these concentrations largely exceeded the amounts of the respective compounds in fresh BB/FCS medium except for glucose (see above). Under the respective conditions of the labeling experiments, a potential reprogramming of the carbon metabolism of *H. pylori* can therefore be envisaged due to the changing substrate availabilities.

### TABLE 1 Concentration of potential carbon substrates in BB/FCS. To account for protein- and peptide-bound amino acids, the medium was also hydrolyzed in 6 M HCl

| Metabolite       | Concentration [mM] | Concentration after hydrolysis [mM] |
|------------------|--------------------|-------------------------------------|
| Alanine          | $1.4 \pm 0.3$      | $8.2 \pm 0.3$                       |
| Glycine          | $0.5 \pm 0.1$      | $8.1 \pm 0.2$                       |
| Valine           | $1.3 \pm 0.3$      | $7.3 \pm 0.2$                       |
| Leucine          | $4.1 \pm 0.8$      | $11.1 \pm 0.1$                      |
| Isoleucine       | $1.2 \pm 0.2$      | $4.8 \pm 0.1$                       |
| Proline          | $0.4 \pm 0.1$      | $10.6 \pm 0.1$                      |
| Serine           | $1.1 \pm 0.3$      | $6.5 \pm 1.8$                       |
| Phenylalanine    | $1.5 \pm 0.3$      | $4.6 \pm 0.1$                       |
| Aspartate        | $0.4 \pm 0.1$      | $13.4 \pm 0.2$                      |
| Glutamate        | $1.1 \pm 0.2$      | $20.1 \pm 0.2$                      |
| Lysine           | $1.2 \pm 0.1$      | $7.1 \pm 0.4$                       |
| Tyrosine         | $0.5 \pm 0.1$      | $2.8 \pm 0.1$                       |
| Glycerol         | $0.5 \pm 0.3$      | n.d.                                |
| Succinate        | $0.1 \pm 0.1$      | n.d.                                |
| Citrate          | $0.0 \pm 0.0$      | n.d.                                |

Note: Standard deviations are based on six values (2 × biological replicates and 3 × technical replicates each).

Abbreviation: n.d. = not determined.

2.2 | $[U-^{13}C_6]$Glucose is efficiently metabolized via the ED-pathway providing labeled glyceraldehyde, pyruvate and acetyl-CoA for fatty acid biosynthesis

Glucose was already reported as a main nutrient used by *H. pylori* growing on horse blood agar plates (Mendz et al., 1993a). To test this finding for *H. pylori* growing in BB/FCS, we added $[U-^{13}C_6]$glucose to this medium at a final concentration of 10 mM and grew *H. pylori* for 15 hr until the mid-exponential growth phase. A representative growth curve is shown in the supplementary information (Figure S1). Bacteria were harvested and GC–MS analysis of multiple metabolites served to elucidate the uptake and metabolic pathways for glucose usage. From cytosolic metabolites obtained via mechanical disruption of the cells in methanol, $^{13}$C-excess and isotopologue profiles for 24 metabolites were determined (for numerical values, see Table S1). The most important results are summarized in Figure 1, integrating the detected $^{13}$C-incorporation of key metabolites into a best possible model for the central carbon metabolism of *H. pylori*. This model was designed on the basis of enzyme activities as expected from the reported genome of *H. pylori* P12 in the KEGG database (Fischer et al., 2010) and recent genome annotations (Kanehisa et al., 2016; Karlsson et al., 2016; Resende et al., 2013).

Efficient uptake and degradation of glucose via the ED-pathway and/or the PPP was reflected by the $^{13}$C-excess of 47% in the predominant M + 3 species in glyceraldehyde, i.e., the isotopologue carrying three $^{13}$C-atoms obtained by conversion of $[U-^{13}C_6]$glucose into $[U-^{13}C_3]$glyceraldehyde phosphate (GAP) and further to $[U-^{13}C_3]$
glyceraldehyde (i.e., M + 3) either by a phosphatase or chemical hydrolysis during sample processing.

Tyrosine is constructed in the shikimate pathway from erythrose 4-phosphate (E-4-P) originating from the PPP, as well as phosphoenolpyruvate (PEP), which is produced from GAP. 13C-Excess in tyrosine is therefore another indicator of active glucose metabolism via the ED-pathway and the PPP. Indeed, tyrosine displayed significant 13C-excess in form of the M + 3 isotopologue (1.3%) and the M + 4 isotopologue (2.3%). This pattern was in line with an active shikimate pathway using M + 3 13C-labeled PEP derived from [U-13C3]GAP and M + 4 E-4-P, produced in the PPP from [U-13C3]glucose.

Phenylalanine biosynthesis also proceeds via the shikimate pathway. In contrast to tyrosine, phenylalanine did not acquire significant 13C-excess > 0.5% (Table S1). Similarly, valine, leucine, isoleucine and methionine were also unlabeled from [U-13C3]glucose. This was expected as these amino acids are essential for H. pylori growth and their respective biosynthetic pathways are not or only partially annotated in the genome of H. pylori (Nedenskov, 1994; Reynolds et al., 1994). Also, alanine, proline and glycine did not display significant 13C-excess, even though H. pylori would have been capable of synthesizing them based on the genome annotations. However, as shown above, these amino acids were abundant in free as well as in bound form in the BB/FCS medium and were presumably taken up (in unlabeled form) from the environment. Looking at the physiological environment of H. pylori, the gastric juice of H. pylori-infected individuals also contains high amounts of proline and alanine (Nagata et al., 2003). Additionally, proline and glycine are the main components of the amino acid backbone in the collagen structure (Shoulders & Raines, 2009), and H. pylori has been shown to secrete a collagenase upon infection, which is essential for colonization in vivo (Kavermann et al., 2003). The abundance of these substrates in the ecological niche of H. pylori might therefore explain the reduced preference for their synthesis as an adaption to this niche.

In serine, only the M + 1 isotopologue was observed with a 13C-excess of 0.9%, but not the M + 3-isotopologue that would have indicated serine biosynthesis via [U-13C3]3-phosphoglycerate and phosphohydroxypyruvate. This was also expected from genomic data where a gene for phosphohydroxypyruvate transaminase is not annotated. The observed M + 1 isotopologue in serine rather arose through a C4-fixation pathway starting from unlabeled glycine and a 13C5-source. Most likely, 13C5-formate is generated as a product from [U-13C5]pyruvate by mixed acid fermentation affording formate and acetate (Mendz et al., 1994b). 13C-Formate could then be converted by a 10-formyltetrahydrofolate amidohydrolase into 13C1-10-formyltetrahydrofolate, which can further be reduced to 13C1-5,10-methylene-5,6-7,8-tetrahydrofolate (MTHF) serving as the precursor for serine catalyzed by serine hydroxymethyltransferase.

The production of labeled lactate from [U-13C3]glucose corroborated an active lactate dehydrogenase (Mendz et al., 1993b). Indeed, the dominant isotopologue was the M + 3 species (4.9%) which can be explained by the lactate dehydrogenase reaction, i.e., it is derived from [U-13C3]pyruvate. Interestingly, there was also a significant fraction of M + 2 present in lactate (2.2%). Analysis of the fragmentation patterns in the mass spectrum of silylated lactate, i.e., by comparison of the mass fractions in fragments comprising C1-C3 or C2-C3 of lactate, revealed similar amounts of [1,2-13C2], and [2,3-13C2]lactate. The formation of [1,2-13C2]lactate suggested an oxaloacetate decarboxylating enzyme that converts [1,2-13C2]oxaloacetate into [1,2-13C2]pyruvate (cf. Figure 1, enzyme 5). However, PEP carboxykinase or oxaloacetate decarboxylase are not annotated in the genome of H. pylori. Therefore, an alternative non-classical oxaloacetate decarboxylating enzyme must be active. Most probably, this reaction is catalyzed by a (4S)-4-hydroxy-2-oxoglutarate aldolase annotated in the genome (enzyme 5 in Figure 1). Indeed, this enzyme is known to produce pyruvate from oxaloacetate (Nishiha et al., 1972), thereby explaining the formation of [1,2-13C2]pyruvate from [1,2-13C2]oxaloacetate. [1,2-13C2]Pyruvate could then be reduced to [1,2-13C2]lactate (cf. Figure 1). The formation of [2,3-13C2]lactate suggested additional carbon fluxes via the reversible PFOR (enzyme 1, Figure 1) (St Maurice et al., 2007) starting from [U-13C2]acetyl-CoA. This reaction yields [2,3-13C2]pyruvate, which is reduced to [2,3-13C2]lactate.

However, the main role of formed acetyl-CoA via PFOR (enzyme 1 in Figure 1) (St Maurice et al., 2007) seemed to be its function as the substrate for fatty acid synthesis as reflected by significant 13C-excess in evenly (9.4%) and unevenly (4.0%) labeled isotopologues of myristate (see Figure 1), which represents the major component in lipid extracts from H. pylori (Inamoto et al., 1995). The isotopologue profiles of all detected fatty acids displayed mostly even-numbered labeled isotopologues using 13C2-acetyl-CoA as the precursor, but also a significant portion of odd label, indicating the usage of M + 1-labeled acetyl-CoA for fatty acid synthesis as well. The formation of this isotopologue can be explained by fluxes involving the citrate cycle and action of enzymes 5 and 1, respectively (cf. Figure 1; green colored). For example, following this route, [1,2-13C2]oxaloacetate could be converted into [1,2-13C2]pyruvate and further into [1,3-13C2]acetyl-CoA.

To further examine the architecture of the TCA cycle, we analyzed citrate, fumarate, malate and succinate as direct intermediates as well as aspartate and glutamate (Figure 1, Table S1). These amino acids can be used as approximations for oxaloacetate and α-ketoglutarate, respectively (Eisenreich et al., 2006). The 13C-incorporation into these compounds was much lower in comparison to glyceraldehyde (see Table S1 and Figure S1). This immediately suggests that the TCA cycle was mainly fueled by unlabeled substrates rather than from the labeled glucose (see also below).

Nevertheless, the observed low 13C-enrichments and profiles still allowed some insights into the fluxes through the TCA cycle (cf. Figure 1). Citrate and free glutamate mainly displayed the M + 1 and M + 2 isotopologues, reflecting the usage of 13C2- and 13C1-acetyl-CoA for the formation of citrate in the TCA-cycle and its conversion into α-ketoglutarate/glutamate. The presence of the M + 3 and M + 4 isotopologues at lower abundances indicated that oxaloacetate, the other precursor for citrate, also carried 13C-atoms (Table S1). The labeling pattern of glutamate generally corresponded
well to succinate and the other TCA cycle intermediates, leading to significant fractions of the M + 2 and M + 1 isotopologues in succinate, fumarate, malate, and oxaloacetate/aspartate, respectively. Interestingly, all C₃-compounds also showed minor amounts of the M + 3 isotopologues (Table S1). Since H. pylori appears to lack enzymes converting M + 3 pyruvate or PEP into oxaloacetate or malate, this was indicative of an alternative anaplerotic route and will be further discussed below with the experiment using ¹³C-bicarbonate. As the M + 3 isotopologue was most prominent in malate, it was tempting to speculate that ¹³C₃-malate could arise in a reaction catalyzed by malate synthase combining M + 2 or M + 1 labeled acetyl-CoA with labeled glyoxylic acid. Notably, earlier in vitro enzyme assays reported the presence of a malate synthase reaction in cell extracts of H. pylori (Pitson et al., 1999) (see also below).

We also analyzed the ¹³C-excess and isotopologue distribution of 13 different proteinogenic amino acids, which were obtained after acidic hydrolysis (Table S11). Here, the values mostly matched the ones of free amino acids in the cytosolic fraction of H. pylori underlining the expected metabolic and isotopic steady state conditions in our experimental setting. The similarity in terms of isotopologue composition between protein-bound and free amino acids validated the experimental protocol as it excludes metabolic alterations due to sample processing. Such alterations would translate within minutes into the isotopologue composition of cytosolic amino acids (Buescher et al., 2015), leading to differences compared to their bound equivalents, which were not observed. The labeling pattern of lysine could only be observed after protein hydrolysis. Here, the observed M + 3, M + 2 and M + 1 isotopologues indicated the well-known biosynthetic pathway using the detected ¹³C-specimens in aspartate and pyruvate as precursors.

2.3 | [U-¹³C₃]Glycerol is not metabolized by H. pylori

To eventually gain more insight into the downstream reactions of carbohydrate degradation, [U-¹³C₃]glycerol was supplied to H. pylori (Table S5). Glycerol could have entered the PPP via its transformation to glyceraldehyde 3-phosphate. However, besides efficient uptake of the substrate resulting in 54% ¹³C-excess in glycerol reisolated from the cytosolic fraction, no significant incorporation into other metabolites was detected. Therefore, glycerol did not serve as a major carbon substrate under the experimental conditions. However, we could not rule out that exogenous glycerol was used as a building block for the glycerol moiety in glycerolipids, which were not among the metabolites under study.

2.4 | [U-¹³C₂]Serine is efficiently utilized and the labeling data support the metabolic model derived from the glucose experiment

Serine, a major component of gastric juice, was reported, next to glucose, as another main carbon source for H. pylori (Nagata et al., 2007). In line with this hypothesis, addition of serine increased the respiratory activity of H. pylori under in vitro conditions (Nagata et al., 2003). Two possible uptake systems for serine exist in H. pylori, SdcA specifically for L-serine, and DagA for D-serine as well as for D-alanine and glycine (Tomb et al., 1997). Indeed, the high ¹³C-excess of ~63% in free serine from the polar fraction (Figure S2, Table S2) indicated efficient uptake of the [U-¹³C₃]serine supplement in our experimental setting. The label distribution in other metabolites showed similarities to the experiment with [U-¹³C₆]glucose (Figure 1, Table S1) since both precursors afford [U-¹³C₃]pyruvate as a common intermediate. This was not unexpected, since [U-¹³C₃]serine is directly converted to M + 3 pyruvate via serine dehydratase, and the detection of the M + 3 isotopologues in lactate (3.5%) and alanine (0.5%) derived from pyruvate confirmed this route. Here, lactate showed significant ¹³C-excess of 8.6%, but the isotopologue profile differed significantly from the one in serine, although the carbon skeleton should remain unchanged through the transformation from serine to lactate via pyruvate. The M + 1 (2.6%) and M + 2 (4.2%) isotopologues in lactate were much more prevalent than in serine. This indicated that a higher fraction of lactate was produced from pyruvate arising through decarboxylation of oxaloacetate (enzyme 5, Figure S2), and not via deamination of serine. At the level of M + 3 pyruvate, the downstream reactions followed the pathways described above for the labeling experiments with [U-¹³C₆]glucose. Indeed, transformation of M + 3-labeled pyruvate yielded M + 2 acetyl-CoA, fatty acids and TCA cycle-related metabolites at low but significant ¹³C-excess (Figure S2). Additionally, and in contrast to the glucose experiment, significant ¹³C-excess was found in M + 2 glycine (17.6%), providing evidence for an active serine hydroxymethyltransferase (see also above) forming ¹³C₂-glycine from ¹³C₂-serine (Figure S2).

Compared to the glucose labeling experiment, with no detectable ¹³C-enrichment in free alanine, there was now a low but significant ¹³C-excess in form of the M + 3 isotopologue (0.5%). This could be due to the action of amino acid uptake by Dgra, which is capable of the uptake of serine, alanine and glycine. In contrast to the glucose experiment, the addition of large amounts of ¹³C-serine to the medium might raise the need for de novo alanine synthesis, as the Dgra uptake system (also capable of alanine uptake) could be saturated by the abundant serine supply and the uptake of alanine was therefore hampered. The proteinogenic amino acids (Table S12) showed again similar excess values and isotopologue distribution as the ones from the cytosolic fraction.

2.5 | Use of ¹³C-bicarbonate shows only minor carboxylating activities in H. pylori and excludes classical anaplerotic reactions

To investigate the usage of bicarbonate in anaplerotic reactions and, generally, to get more insights into the capnophilic nature of H. pylori (Bury-Mone et al., 2006), we grew H. pylori in BB/FCS medium containing 50 mM ¹³C-bicarbonate. In a similar setting, the efficient
usage of $^{13}$C-bicarbonate was shown for *C. jejuni*, a close relative to *H. pylori* (Gao et al., 2017). Notably, gastric extracts contain 25 mM bicarbonate, thus providing a potential (additional) carbon source for *H. pylori* (Feldman, 1983).

In our labeling experiment, $^{13}$C-bicarbonate did not produce $^{13}$C-excess in the metabolites under study, except for minor enrichments (<2%) in citrate, glycerol and serine as $M + 1$ isotopologues (Table S6). The data suggested that a minor fraction of $M + 1$ $^{13}$C-pyruvate was generated through carboxylation of acetyl-CoA via PFOR as reported earlier (enzyme 1, as indicated in Figure 1) and using $^{13}$C-bicarbonate as a substrate (Hughes et al., 1995; St Maurice et al., 2007). $M + 1$ $^{13}$C-pyruvate could then be converted into $M + 1$ glycerol and $M + 1$ serine following the routes shown in Figure 1. No evidence was obtained for other CO$_2$ or bicarbonate utilizing pathways, in particular producing labeled acetyl-CoA, which would have been detected in labeled fatty acids or TCA cycle intermediates. Notably, fatty acids and TCA cycle intermediates (except for a minor fraction of $^{13}$C-citrate, see below) were devoid of $^{13}$C-label in this experiment.

This finding also indicated that *H. pylori* did not use reactions indicative for the reductive TCA cycle via citrate lyase or its variant via citrate synthase (Mall et al., 2018), the 3-hydroxypropionate/4-hydroxybutyrate cycle or the dicarboxylate/4-hydroxybutyrate cycle (Hügler et al., 2011), thus confirming the metabolic model shown in Figure 1. The small amount of $M + 1$ label present in citrate (0.8%) could be explained by a reversible isocitrate dehydrogenase in line with the results of the $[U-^{13}$C$_5]$glutamate experiment (see below).

Looking at the genomes of the 61 *H. pylori* strains in the KEGG database (Kanehisa et al., 2016), including *H. pylori* P12 used in this study, all of them lack any form of phosphoenolpyruvate carboxylase or pyruvate carboxylase. In contrast, all other members of the family of *Helicobacteriaceae*, of which the genomes are sequenced, including other *Helicobacter* species, *Sulfurimonas denitrificans* (Sievert et al., 2008), *Wolinella succinogenes* (Baar et al., 2003), *Sulfurcurvum kujiense* (Han et al., 2012), and *Sulfurovum lithotrophicum* (Jen et al., 2017), show the genomic sequence for at least one of these carboxylating enzymes. *Helicobacter acynonychis* is the only exception, but was shown to be the closest relative to *H. pylori* when analyzing the 16S and 23S rRNA genes of 55 *Helicobacter* species (Dewhirst et al., 2005), and is supposed to have originated from *H. pylori* after a host jump (Epping et al., 2006). Looking further into the order of *Campylobacterales*, anaplerotic enzymes also appear as a common feature. Moreover, other extracellular pathogens like enterohemorrhagic *Escherichia coli* (Perna et al., 2001), *Pseudomonas aeruginosa* (Stover et al., 2000) or *Staphylococcus aureus* (Kuroda et al., 2001) show the ability to replenish the TCA cycle via carboxylation reactions. Therefore, the lack of “classical” anaplerosis seems to be a specific feature of *H. pylori* metabolism probably resulting from the coevolution with its human host for at least 88,000 years (Moodley et al., 2012). This coevolution led to a high level of adaptation of *H. pylori* to its ecological niche (Alloul et al., 2019; Atherton & Blaser, 2009). This is also apparent by the reduced genome size of *H. pylori* (~1.68 Mbp) compared to *W. succinogenes* (~2.1 Mbp).

More specifically, *H. pylori* probably adapted to utilize a variety of substrates from its environment without the need for an efficient C1-anaplerosis using either bicarbonate or CO$_2$–metabolic traits that probably go back to the early evolution and origin of life (Fuchs, 2011; Weiss et al., 2016). Rather, the finding that CO$_2$ or bicarbonate are not relevant in the metabolic network of *H. pylori* supported the hypothesis that the capnophilic nature of *H. pylori* is related to non-metabolic cellular functions like the control of acetyl-CoA carboxylase (Burns et al., 1995), buffering of the periplasmic pH through carbonic anhydrase (Marcus et al., 2005), or suppressing the stringent response (Park et al., 2011).

### 2.6 | $[U-^{13}$C$_4]$Glutamate or $[U-^{13}$C$_5]$succinate is efficiently metabolized via a closed TCA cycle, with rapid equilibrium fluxes between $\alpha$-ketoglutarate and citrate

As discussed above, $^{13}$C-glucose was efficiently used in the ED-pathway but yielded comparably low $^{13}$C-incorporation into TCA cycle related metabolites. This suggested that the cycle had to be fueled by additional carbon sources without the involvement of C1-anaplerosis (see above). To identify these carbon sources and to further elucidate the carbon fluxes in the TCA cycle, we used $[U-^{13}$C$_4]$glutamate or $[U-^{13}$C$_5]$succinate as supplements to *H. pylori* growing in BB/FCS.

The high excess in free glutamate (46%) isolated from the polar fraction of bacteria supplemented with $[U-^{13}$C$_5]$glutamate suggested its efficient uptake. Quite obviously, $[U-^{13}$C$_5]$glutamate entered the TCA cycle via transamination to $[U-^{13}$C$_5]$α-ketoglutarate (Figure 2). Consequently, succinate and citrate displayed high $^{13}$C-excess values in terms of the M + 4 (17.3%) or M + 5 isotopologues (10.9%), respectively. A typical α-ketoglutarate dehydrogenase is absent in *H. pylori* (Pitson et al., 1999). However, the production of M + 4 succinate from $[U-^{13}$C$_5]$α-ketoglutarate/glutamate can be explained by an α-ketoglutarate oxidoreductase producing $^{13}$C$_4$-succinyl-CoA (enzyme 2 in Figure 2) (Hughes et al., 1998). $^{13}$C$_4$-Succinyl-CoA is then converted to $[U-^{13}$C$_5]$succinate via a known CoA-transferase of *H. pylori* (enzyme 3 in Figure 2), which uses acetocacetate as the CoA-acceptor (Corthesy-Theulaz et al., 1997). Further propagation of M + 4 succinate through the TCA cycle produced the predominant M + 4 isotopologues in fumarate, malate and aspartate.

Formation of M + 5 labeled citrate suggested a rapid equilibrium between citrate and glutamate through the action of a reversible isocitrate dehydrogenase leading to $[U-^{13}$C$_5]$aconitate by reaction of $[U-^{13}$C$_5]$α-ketoglutarate with unlabeled bicarbonate/CO$_2$ (Buchanan et al., 2017; Kanao et al., 2002). Aconitase was already shown to act reversibly in *H. pylori* (Pitson et al., 1999), thus affording the detected M + 5 $^{13}$C-citrate from $[U-^{13}$C$_5]$aconitate. Further reaction via an active citrate lyase or reverse citrate synthase reaction producing acetyl-CoA and oxaloacetate can be excluded as utilization of M + 5 citrate would produce M + 3 labeled aspartate, malate and fumarate, but not the observed predominant M + 4 labeled specimens.
of these metabolites. The apparent equilibrium between labeled α-ketoglutarate and labeled citrate supported carbon fluxes from α-ketoglutarate in the reverse direction of the TCA cycle potentially filling up the pools for isocitrate (required as a substrate for the isocitrate lyase reaction, see below) or the generation of NADPH in the reverse isocitrate dehydrogenase reaction (required e.g. as the oxidant in the glucose 6-phosphate dehydrogenase reaction of the ED-pathway). Alternatively, it is also tempting to speculate that citrate is the desired product via this route, which might then be secreted and used as a signal for host cells. To further check this hypothesis, we analyzed the amount of citrate in the medium by GC–MS from the growth experiment with [U-13C5]glutamate as well as with [U-13C2]glycine, [U-13C3]serine, or [U-13C4]aspartate for comparison (Table S23). However, the detected reverse flux caused by 13C-glutamate in the TCA cycle did not lead to increased levels of citrate or isocitrate in the supernatant as compared to the other experiments. Nevertheless, it cannot be excluded that secretion of citrate could be relevant when *H. pylori* thrives in the human host environment.

[U-13C4] Succinate was also efficiently taken up and metabolized, thereby producing similar labeling patterns in aspartate, fumarate and malate as in the experiment with [U-13C5]glutamate (Figure S3). Besides succinate from the cytosolic fraction, malate had the highest...
13C-excess (31.6%) followed by fumarate (23.4%) and aspartate (11.8%) (Table S8). The data did not support a reverse α-ketoglutarate oxidoreductase since the fraction of the M + 4 isotopologue in glutamate was very low (1.2%). Citrate showed a diverse labeling pattern with M + 4 as the main isotopologue. The formation of this isotopologue starting from either [U-13C4]glutamate or [U-13C4]succinate provided strong evidence for fluxes via a closed oxidative TCA cycle as the complete 13C-backbone of the respective tracers remained intact throughout the cycle. Photometric measurements in H. pylori cell extracts also support the activity of a succinate oxidizing enzyme (Chen, 1999; Lancaster & Simon, 2002). However, it is still unclear whether the fumarate reductase in H. pylori can act in a reversible fashion, as proposed earlier (Ge et al., 2000), or whether another unidentified enzyme is capable of succinate oxidation.

2.7 | Labeling profiles produced from [U-13C4]aspartate support the closed TCA cycle topology

In earlier experiments, it was shown that the addition of aspartate increases the growth rate of H. pylori during co-incubation with gastric epithelial cells (van Amsterdam & Ende, 2004). On the other hand, aspartate did not increase the respiratory activity when added during in vitro cultivation (Nagata et al., 2003). However, in our labeling experiment with [U-13C4]aspartate, we observed very high 13C-excess especially in TCA cycle related metabolites, which are indirectly connected to respiratory activity (Table S3, Figure S3). More specifically, malate, succinate and fumarate reflected efficient incorporation of [U-13C4]aspartate by M + 4 isotopologues above 26% (Figure 3). Besides transamination of aspartate to oxaloacetate, aspartate can be converted into fumarate by the catalytic action of aspartate ammonia lyase, a well-known reaction in H. pylori (Mendez & Hazell, 1995). Minor amounts of the M + 3 species (about 3%, Table S3) in these metabolites could be due to an active malate synthase reaction combining M + 1 or M + 2 labeled acetyl-CoA and glyoxylate (see below).

Additionally, citrate and glutamate were also significantly labeled, as well as fatty acids (Table S3). The predominant M + 4 (citrate) or M + 3 (glutamate) isotopologues in these metabolites arose through the conversion of 13C4-aspartate to M + 4 oxaloacetate, which was subsequently used by the citrate synthase affording M + 4 citrate. Subsequently, M + 4 citrate was transformed into M + 3 α-ketoglutarate and the detected M + 3 glutamate. Accordingly, the heavier isotopologues resulted from combinations of M + 4 oxaloacetate and M + 1/M + 2 acetyl-CoA. The production of M + 3 pyruvate/alanine as well as M + 2 acetyl-CoA/fatty acids again corroborated the activity of an oxaloacetate decarboxylating enzyme, most likely of the (4S)-4-hydroxy-2-oxoglutarate aldolase (see above; enzyme 5 in Figure 3).

The proteinogenic amino acid fraction (Table S13) also showed significant 13C-excess in aspartate, glutamate and threonine. Additionally, alanine, lysine and tyrosine displayed 13C-excess values ranging from 1.5% to around 10%. These profiles matched the data of free amino acids from the polar fraction. The isotopologue composition of lysine was dominated by M + 3 (3.7%) and M + 4 (4.0%) species, while tyrosine mostly consisted of M + 1, M + 2- and M + 3-labeled molecules (0.8%). In terms of lysine, this indicated the usage of fully labeled aspartate (M + 4) or pyruvate (M + 3) as precursors, while tyrosine was synthesized with differently labeled PEP as a building block. The absence of significant amounts of heavier isotopologues (> M + 3) in tyrosine reflected that labeled E-4-P was not produced via gluconeogenesis and the PPP at significant rates (cf. Figure 3).

2.8 | [U-13C4]Aspartate leads to rapid equilibrium fluxes between oxaloacetate and succinate and subsequent succinate secretion

When supplementing [U-13C4]aspartate, succinate displayed an anomaly in terms of 13C-excess and isotopologue pattern. Its overall 13C-excess was higher (32%) than in glutamate (8%) and the labeling pattern of succinate only partially resembled the one of glutamate. On this basis, it can be assumed that succinate was not only formed from aspartate/oxaloacetate via the oxidative TCA cycle and α-ketoglutarate/glutamate as its direct precursor. Rather, the high 13C-enrichment in succinate could be caused by the reaction of the aspartate ammonia lyase producing M + 4 fumarate (see also above) and further M + 4 succinate by the fumarate reductase. This reaction path together with aspartate assimilation through transamination to oxaloacetate is depicted in blue in Figure 3.

To account for eventual succinate excretion due to this reverse carbon flux in the TCA cycle, we analyzed the amount of succinate in the medium after growth with [U-13C4]aspartate and [U-13C4]glutamate/[U-13C4]glycine/[U-13C4]serine/[1,5,13C2]citrate for comparison. Indeed, GC–MS-quantification revealed a 4–9-fold increase in the concentration of medium succinate after growth with 13C-aspartate as compared to other 13C-substrates, while the isotopologue profile of secreted succinate was similar to cellular succinate (Table S23).

This reverse flow in the TCA cycle probably allows for an active fumarate respiration (Ge et al., 2000), which is energetically less efficient than using O2 as the terminal electron acceptor. Similarly, aspartate uptake also leads to increased fumarate respiration in Salmonella typhimurium (Nguyen et al., 2020). Active fumarate respiration upon aspartate usage could also explain the discrepancy between efficient aspartate metabolization observed in this work and the lack of oxygen consumption after addition of aspartate to H. pylori growing in broth as observed earlier (Nagata et al., 2003). To verify that the apparent active fumarate reduction upon excess of exogenous aspartate was not due to decreasing levels of oxygen during the cultivation, we performed the same experiment with a shortened labeling period of only two hours. After this short period of growth, it can be assumed that the oxygen concentration is still relatively high. The labeling patterns of the TCA cycle intermediates (Table S21) resembled the ones of the 8-hr labeling experiment, although the 13C-excess values were generally lower. Thus, there is no indication of
a biphasic growth behavior with a general reversal of carbon flux in the TCA cycle during growth of *H. pylori* under these conditions.

2.9 Evidence for reactions via a full or partial glyoxylate bypass, and possible pathways for the generation of glyoxylate

The labeling experiments with $^{13}$C-aspartate and $^{13}$C-glutamate demonstrated that both amino acids efficiently serve to fill up the TCA cycle of *H. pylori*. However, the lack of classical anaplerotic reactions raised the question of alternative metabolic pathways to replenish TCA cycle intermediates under conditions where aspartate or glutamate are not available from the environment in sufficient amounts. The need for the presence of such a metabolic pathway also becomes apparent since aspartate, asparagine, glutamate and glutamine are non-essential for *H. pylori* (Reynolds & Penn, 1994).

In the following, we provide some evidence for the malate synthase reaction as a possible alternative for filling up intermediates in the TCA cycle. This reaction could allow the formation of malate...
solely from acetyl-CoA, a metabolite that can be produced from various sources e.g. by degradation of glucose or amino acids. One molecule of acetyl-CoA is directly utilized in the malate synthase reaction, while the carbon skeleton of glyoxylate could also potentially come from acetyl-CoA via the citrate synthase and subsequent isocitrate lyase reaction. A corresponding gene for a malate synthase is not assigned in the genome of H. pylori, but its activity was detected by genome annotations or BLAST search despite indications for production of labeled pyruvate since lactate and alanine were not labeled by 13C-excess (57%) in re-isolated glycine from the polar fraction, but its activity was detected in H. pylori supernatants via 1H-NMR as well as in photometric assays (Hoffman et al., 1996; Pitson et al., 1999).

To experimentally substantiate an active malate synthase in a potential glyoxylate bypass, leading to the specific 13C-incorporations described above, we tested different possible origins of glyoxylate in experiments using [U-13C]acetate, [1,5-13C]citrate, [U-13C]proline, or [U-13C]glycine as precursors. In case of an active isocitrate lyase, [1-13C]glyoxylate would be generated from [1,5-13C2]citrate (Dolan & Welch, 2018). [U-13C]Proline could be converted to 13C2-pyruvate and 13C2-glyoxylate via a pathway that is only partially annotated in H. pylori (Nishiara & Dekker, 1972; Watanabe et al., 2012). [U-13C]Glycine could lead to [U-13C]glyoxylate via an aminotransferase that has not been described in H. pylori so far (Nishiyia & Imanaka, 1998). Additionally, glycine could also be used via the β-hydroxyaspartate cycle, recently found in some marine proteobacteria. Herein, the combination of glycine and glyoxylate would lead to aspartate/oxaloacetate and subsequently to malate as an alternative to the malate synthase reaction (Schada von Borzyskowski et al., 2019). Finally, [U-13C]acetate could be transformed to acetyl-CoA, which, amongst other pathways, could serve as a starting substrate for the glyoxylate bypass or, alternatively for the ethylmalonyl-CoA pathway (Erb et al., 2009). Via this pathway, 13C2-glyoxylate would be generated retaining the carbon skeleton of the acetyl-moiety in acetyl-CoA (Peyraud et al., 2009). All these potential pathways for the generation of glyoxylate are summarized in Figure 4.

### 2.10 | [U-13C2]Proline, [U-13C2]glycine, and [U-13C2]acetate are excluded as potential precursors for glyoxylate formation

[U-13C2]Glycine uptake was high as gleaned from the detected 13C-excess (57%) in re-isolated glycine from the polar fraction, but its metabolism only yielded significant 13C-excess in serine with the M + 2-isotopologue being the most prominent species (Table S9). No labeling was found in aspartate or other TCA cycle related metabolites. On this basis, an active β-hydroxyaspartate cycle as well as the formation of glyoxylate from glycine can be ruled out for H. pylori. [U-13C2]Proline was mainly converted to [U-13C2]glutamate and to downstream products as described above. However, there was no indication for production of labeled pyruvate since lactate and alanine did not show significant 13C-incorporation (Table S10). Accordingly, no labeled glyoxylate was produced from [U-13C2]proline. [13C2]Acetate only led to small 13C-excess values in fatty acids and citrate (Figure S4). Exogenous acetate can therefore also be excluded as origin of a potential glyoxylate bypass. The ethylmalonyl-CoA pathway can also be excluded on this basis (Table S22).

### 2.11 | The labeling patterns from [1,5-13C2]citrate as well as a photometric assay support an active isocitrate lyase as the source of glyoxylate

Metabolization of [1,5-13C2]citrate led to M + 2 glutamate (5.0%) and M + 1 succinate (5.1%, Figure S5). Further propagation through the TCA cycle led to M + 1 maleate (2.7%) and aspartate (1.8%). GC-MS-analysis of the mass distributions in the fragments of aspartate indicated an equal distribution of the 13C-label between the C1- and C4-positions. This suggests that aspartate/oxaloacetate is formed from citrate through symmetrical intermediates, such as succinate (Table S7). Although this pattern could generally be well explained by a cyclic TCA cycle structure, it is notable that the 13C-excess in maleate was significantly higher than in fumarate. It is therefore tempting to speculate that [1,5-13C2]citrate was converted into [1,5-13C2]isocitrate, and subsequently into [1-13C]succinate and [13C2]glyoxylate via the isocitrate lyase reaction, followed by the malate synthase reaction to yield 13C1-malate from [1-13C]glyoxylate (Figure 4 D). Formation of [1-13C]succinate leads to randomization of the label through the terminal positions (i.e., C-1 and C-4) in the downstream products fumarate, maleate, oxaloacetate/aspartate, as detected in aspartate. On the other hand, starting from [13C2]glyoxylate the malate synthase reaction yields [1-13C]malate, which could be directly converted to [13C2]oxaloacetate/aspartate and not into the observed 1:1 mixture of [1-13C] and [4-13C]aspartate. However, the finding of equal 13C-incorporation into C-1 and C-4 of aspartate in the experiment with [1,5-13C2]citrate does not exclude the glyoxylate hypothesis since label randomization could also have occurred by the highly active fumarase in H. pylori establishing a rapid equilibrium between the malate and fumarate pools with loss of the positional 13C-labeling information in malate and its downstream products (Pitson et al., 1999).

As already mentioned above, the glyoxylate bypass hypothesis is challenged by the fact that a gene for isocitrate lyase is not assigned in the genome of H. pylori. To provide direct experimental evidence for a non-conventional isocitrate lyase in H. pylori, we performed photometric assays using crude cell extracts of H. pylori. Non-conventional here means that the enzyme was so far not detectable by genome annotations or BLAST search despite indications for its activity. Herein, H. pylori P12 cells were disrupted by ultra-sonification and the supernatants were used in enzyme assays containing aspartate as a potential substrate. Here, the formation of glyoxylate is monitored photometrically via the formation of the phenyldihydrazone of glyoxylate. As a positive control, we used cell extracts of E. coli possessing the enzymes of a glyoxylate pathway including isocitrate lyase (Chung et al., 1988). As shown in Figure 5, the photometric test with cell extracts of H. pylori was positive with a similar signal intensity for glyoxylate formation as observed for the E. coli extract. This activity was dependent on isocitrate and magnesium ions in the assay. Together, this provided substantial evidence...
for a Mg\textsuperscript{2+}-dependent activity of isocitrate lyase or an isocitrate lyase like enzyme in cell extracts of \textit{H. pylori}. Several other \textit{H. pylori} strains were tested and showed the same isocitrate-dependent activities. As another control, an \textit{E. coli} ΔaceA mutant lacking the gene for isocitrate lyase did not show the same activity in this assay, excluding potential unspecific reactions in the supernatant leading to a false positive result (Figure 5). Together with earlier reports for malate synthase activity in \textit{H. pylori} (Hoffman et al., 1996; Pitson et al., 1999), the labeling experiments using [1,5-\textsuperscript{13}C\textsubscript{2}]citrate as well as the detected activity of a non-conventional isocitrate lyase supported a functional glyoxylate bypass in \textit{H. pylori}.

It is currently unknown which enzymes catalyze the reaction of a putative glyoxylate bypass in \textit{H. pylori}. As mentioned above, there are no indications for either malate synthase or isocitrate lyase in the genome of \textit{H. pylori}. More specifically, BLAST searches using various sequences of isocitrate lyase or malate synthase from other organisms (\textit{e.g.} \textit{E. coli}, \textit{P. aeruginosa}, other ε-proteobacteria) did not retrieve any weak orthologs in the \textit{H. pylori} genome. This suggests that these reactions are carried out in \textit{H. pylori} by enzymes that developed substrate promiscuity or changed their substrate specificity during adaption of \textit{H. pylori} to the human host, similar to (4S)-4-hydroxy-2-oxoglutarate aldolase catalyzing oxaloacetate decarboxylation in \textit{H. pylori}.

**Figure 4** Potential origins of glyoxylate, which are examined by labeling experiments. Red dots indicate \textsuperscript{13}C-label in the substrates as well as potential \textsuperscript{13}C-incorporation in the expected products. (A) Production via proline degradation was tested with [U-\textsuperscript{13}C\textsubscript{5}]proline. (B) Transamination of glycine was tested with [U-\textsuperscript{13}C\textsubscript{2}]glycine. (C) The ethylmalonyl-CoA-pathway was tested with [U-\textsuperscript{13}C\textsubscript{2}]acetate. (D) Production via isocitrate lyase was tested with [1,5-\textsuperscript{13}C\textsubscript{2}]citrate. Blue dots indicate \textsuperscript{13}C-incorporation via the glyoxylate bypass. Mixed colored dots indicate \textsuperscript{13}C-incorporation via the oxidative TCA cycle and/or the glyoxylate bypass. The detected labeling patterns were only in agreement with the predictions via (D).
resulted in an interrupted TCA cycle and increased persister formation during stationary growth (Wang et al., 2018). In the context of persister cells, the inactivation of α-ketoglutarate dehydrogenase subunits resulted in an interrupted TCA cycle and increased persister formation during stationary growth (Wang et al., 2018). In *Burkholderia cepacia* biofilms, downregulation of TCA cycle-related genes led to reduced ROS (reactive oxygen species) production (Van Acker et al., 2013). The balance of carbon usages via an adaptive TCA cycle could therefore reflect a smart toolbox for *H. pylori* to rapidly react to its changing environment and to allow the formation of persister cells.

FIGURE 5  Isocitrate lyase activity is detectable in lysates of different *H. pylori* strains. Bacterial lysates were incubated with (+) or without (w/o) isocitric acid and the formation of glyoxylate-phenylhydrazone was spectrophotometrically assessed at 324 nm. The isocitrate lyase activity of *H. pylori* P12 cell extracts was strongly dependent on the presence of the cofactor Mg²⁺ (left). As expected, no glyoxylate-phenylhydrazone formation was observed in an *E. coli* ΔaceA mutant (middle). Further *H. pylori* strains were tested and showed comparable isocitrate lyase activities (right). Data show means and standard deviations of three independent experiments.

3  |  CONCLUSION

In this work, the central carbon metabolism of *H. pylori* was elucidated using a comprehensive ¹³C-labeling strategy starting from various substrates. On one hand, the data showed that exogenous glucose was efficiently utilized via the ED-pathway and the PPP, leading to triose phosphates, but less efficiently downstream of pyruvate/acetyl-CoA via the TCA cycle for ATP generation. On the other hand, substrates entering the metabolism via the TCA cycle yielded high ¹³C-enrichments in the intermediates of the TCA cycle and related products, but not in metabolites upstream of pyruvate/PEP (Figure 6). This was a clear indication for multiple substrate usage in a bipartite metabolic network in which glucose serves an efficient source to build up bacterial cell walls and lipids, whereas multiple substrates serve to feed the citrate cycle with fluxes determined by the nutrient available from the environment (Figure 6). The dampened TCA cycle activity in response to e. g. glucose could reflect means to support chronic colonization—a hallmark of *H. pylori* infection (Lina et al., 2014)—as it produces less energy but still allows respiration in the absence of oxygen via fumarate as a terminal electron acceptor (Ge, 2002). Further, a connection between a reduced activity of the closed oxidative TCA cycle and persistence becomes more and more apparent in other pathogens (Jung et al., 2019; Trastoy et al., 2018). In *E. coli*, upregulation of fumarate reductase, possibly allowing for more efficient fumarate respiration, led to increased persister formation (Kim et al., 2016), while in *S. aureus* inactivation of α-ketoglutarate dehydrogenase subunits resulted in an interrupted TCA cycle and increased persister formation during stationary growth (Wang et al., 2018). In *Burkholderia cepacia* biofilms, downregulation of TCA cycle-related genes led to reduced ROS (reactive oxygen species) production (Van Acker et al., 2013). The balance of carbon usages via an adaptive TCA cycle could therefore reflect a smart toolbox for *H. pylori* to rapidly react to its changing environment and to allow the formation of persister cells.

With all substrates under study, we observed a closed TCA cycle topology, standing in contrast to earlier reports about a bifurcate TCA cycle structure (Han et al., 2018; Pitson et al., 1999). However, the adaptive fluxes through the TCA cycle observed in our study might produce a bifurcate-like metabolic phenotype under different cultivation conditions thereby explaining the discrepancy. We could show that *H. pylori* reacts to environmental succinate and glutamate with an apparent shift of carbon fluxes. This adaption could have been acquired by *H. pylori* to rapidly respond to immune reactions of its host. Glutamine is the main carbon substrate for immune cells especially during proliferation and, in part, it also acts as a signaling molecule for immune cell activation (Cruzat et al., 2018). *H. pylori* could utilize this extracellular glutamine by secretion of a γ-glutamyltranspeptidase, which converts glutamine to glutamate and thereby makes it accessible to *H. pylori* as a carbon substrate (Shibayama et al., 2007). Similarly, succinate accumulates upon infection in M1 macrophages (O’Neill & Pearce, 2016) and also works as a signaling molecule. Extracellular succinate binds to GPR91 leading to proinflammatory IL-1β expression via HIF-1α (de Castro Fonseca et al., 2016; Fernandez-Veledo & Vendrell, 2019; Tannahill et al., 2013) and increases cytokine production in dendritic cells (Rubic et al., 2008). These functions of succinate and glutamine in the context of immunometabolism also make them abundant and welcome substrates for *H. pylori* when being challenged by immune cells. Scavenging extracellular succinate as well as glutamine could therefore be advantageous for *H. pylori* to deal with immunological reactions of the human host cell. Decreasing the concentrations of these metabolites could reduce proinflammatory responses due to *H. pylori* infection as well as macrophage proliferation, thereby allowing persistent *H. pylori* infection. Additionally, the surplus in energy produced by TCA cycle activation due to glutamate and succinate usage could be beneficial for defense mechanisms, like energy intensive shedding of outer membrane vesicles (OMVs), which protect *H. pylori* against oxidative burst (Kulp & Kuehn, 2010; Lekmeechai et al., 2018).
The observed lack of classical anaplerosis, some anomalies observed in our labeling experiments, and the detected enzymatic activities of malate synthase (Hoffman et al., 1996; Pitson et al., 1999) and isocitrate lyase in cell extracts of *H. pylori* (Hoffman et al., 1996 and this study) suggest the dependency of the citrate cycle on a putative glyoxylate bypass (Figure 6), although there are no homologies for malate synthase and isocitrate lyase in the genome sequences of *H. pylori*. These still uncharacterized enzymes may provide new opportunities for antimicrobial targets especially given the metabolic orthogonality to the human host (Dolan & Welch, 2018), besides other TCA cycle related enzymes such as α-ketoglutarate oxidase and fumarate reductase shown to be essential for *H. pylori* survival (Chen et al., 2012; Tsugawa et al., 2008). Noteworthy, there has already been a considerable amount of research about the glyoxylate shunt and its inhibition in *Mycobacterium tuberculosis* yielding promising drug candidates against either isocitrate lyase or malate synthase (Krieger et al., 2012; Pham et al., 2017).

4 | EXPERIMENTAL PROCEDURES

4.1 | *H. pylori* growth conditions and labeling experiments

*H. pylori* strains P12, G27, 26,695 and Tx30a were cultured on GC agar plates (Oxoid) supplemented with 8% horse serum (Life
Technologies) and 1% vitamin mix under microaerobic atmosphere (85% N₂, 10% CO₂, 5% O₂) at 37°C. For ₁³C-labeling experiments, *H. pylori* P12 grown on plates were diluted to an OD₅₅₀ of 0.1 in Brucella broth (Becton Dickinson)/10% fetal calf serum (FCS, heat inactivated, Life Technologies) and precultured for 8 hr under a microaerobic atmosphere (85% N₂, 10% CO₂, 5% O₂) at 37°C and shaking at 100 rpm. Next, main cultures (50 ml) were inoculated to a final OD₅₅₀ of 0.05 in Brucella broth/10% FCS and supplemented with either 5 mM [U-₁³C₂]L-serine, 5 mM [U-₁³C₂]L-aspartate, 5 mM [U-₁³C₃]D-glutamate, 5 mM [U-₁³C₆]D-glucose, 10 mM [U-₁³C₃]D-succinate, 5 mM [U-₁³C₅]D-pelargonate, respectively (all tracers with 99% ₁³C-content, purchased from Sigma-Aldrich). Cultures were again shaken for 14–16 hr under a microaerobic atmosphere (85% N₂, 10% CO₂, 5% O₂) at 37°C and shaking at 100 rpm until the exponential growth phase. Bacteria were harvested by centrifugation at 4,000 rpm and 4°C for 20 min. Pellets were inactivated by freezing (30 min, dry ice) and subsequent boiling for 20 min. The samples were stored at −80°C and freeze-dried before further analysis. All experiments were done in duplicates. Similarly, main cultures (50 ml) were again inoculated to a final OD₅₅₀ of 0.1 and supplemented with 5 mM [U-₁³C₆]L-aspartic acid, but growth was only allowed for 2 hr. Harvest and further processing of the pellets was done as described above.

### 4.2 Metabolite extraction procedure

An amount of 5 mg of the freeze-dried cell pellet was mixed with 1 ml of cold (4°C) methanol (VWR). After the addition of 800 mg of glass beads (0.25–0.5 mm), mechanical cell lysis was performed using a ribolyser system (Hybaid) for 1 x 20 s at 4.0 ms⁻¹ and for 4 x 20 s at 6.5 ms⁻¹. Afterwards, the sample was centrifuged at 3,200 g for 10 min and the supernatant was dried under a stream of nitrogen. For silylation, 50 µl of anhydrous acetonitrile as well as 50 µl of a solution of 5 mM norvaline in methanol (internal standard) was added and the mixture was incubated at 70°C for 10 min and the supernatant was dried under a stream of nitrogen at 70°C. The residue was treated with 50 µl of a solution of 5 mM norvaline in methanol (internal standard). The mixture was dried under a stream of nitrogen and the residue was treated with 50 µl of tert-butyldimethylsilyl (TBDMS) derivatives (TBDMS) were analyzed by GC–MS after derivatization, TBDMS-arginine could also not be detected in sufficient amounts. Furthermore, acid hydrolysis led to conversion of glutamine and asparagine to glutamate and aspartate, respectively. Therefore, results given for aspartate and glutamate correspond to asparagine/aspartate and glutamine/glutamate, respectively.

### 4.4 Protein hydrolysis

For analysis of protein-bound amino acids, 2 mg of the bacterial sample (lyophilized cell pellet) were suspended in 1 ml of 6 M hydrochloric acid and hydrolyzed for 15 hr at 105°C. The reaction mixture was dried under a stream of nitrogen at 70°C. The residue was suspended in 200 µl of 50% aqueous acetic acid using an ultrasonic bath for 3 min. The solution was applied onto a small column of Dowex 50WX8 (7 x 10 mm; 200–400 mesh, 34–74 µm, H⁺-form, Alfa Aesar). The column was first washed with 2 ml of H₂O, then eluted with 1 ml of 4 M aqueous ammonia. The ammonia eluate was dried under a stream of nitrogen at 70°C. The residue was treated with 50 µl of N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide and 50 µl of anhydrous acetonitrile at 70°C for 30 min. The TBDMS-derivatives of amino acids were again analyzed by GC–MS. Due to degradation of tryptophan, methionine and cysteine during acid hydrolysis, these amino acids could not be analyzed. Due to inefficient derivatization, TBDMS-arginine could also not be detected in sufficient amounts. Furthermore, acid hydrolysis led to conversion of glutamine and asparagine to glutamate and aspartate, respectively. Therefore, results given for aspartate and glutamate correspond to asparagine/aspartate and glutamine/glutamate, respectively.

### 4.5 Gas chromatography-mass spectrometry (GC–MS) analysis

GC–MS analysis was performed with a QP2010 Plus gas chromatograph/mass spectrometer (Shimadzu) equipped with a fused silica capillary column (Equity TM-5; 30 m, 0.25 mm, 0.25 µm film thickness; SUPELCO) and a quadrupole detector working with electron impact ionization at 70 eV. An aliquot (0.1 to 6 µl) of the derivatized samples were injected in 1:5 split mode at an interface temperature of 260°C and a helium inlet pressure of 70 kPa. Selected ion monitoring (SIM) was used with a sampling rate of 0.5 s. LABSOLUTION software (Shimadzu) was used for data collection and analysis. For the measurement of polar metabolites, the column was kept at 100°C for 2 min after sample injection. Following, the column was developed with a first gradient of 3°C min⁻¹ until a final temperature of 234°C. Subsequently, a second temperature gradient of 1°C min⁻¹ until a final temperature of 237°C, and a third temperature gradient of 3°C min⁻¹ to a final temperature of 260°C was performed.

For the measurement of proteinogenic amino acids, the column was held at 150°C for 3 min followed by a temperature gradient of 10°C min⁻¹ to a final temperature of 280°C. Isotopologue calculations were typically performed with molecular fragments at m/z of [M-57]+, where M is the molecular mass of the respective TBDMS-derivative. For analysis of the growth medium, analysis was done in
the SCAN mode using the temperature program from the analysis of polar metabolites with a mass range of 50–700 m/z.

All samples were measured three times to obtain technical replicates. $^{13}$C-Excess values and isotopeologue compositions were calculated as previously described (Eylert et al., 2008). This comprises (i) the detection of GC–MS spectra of unlabeled derivatized metabolites, (ii) determination of the absolute mass of isotopeologue enrichments and distributions of labeled metabolites of the experiment, and (iii) correction of the absolute $^{13}$C-incorporation by subtracting the heavy isotopeologue contributions due to the natural abundances in the derivatized metabolites (Eylert et al., 2008; Lee et al., 1991).

4.6 | Generation of an E. coli aceA mutant

An E. coli aceA deletion mutant was generated according to the protocol established by Datsenko and Wanner (Datsenko & Wanner, 2000). The DNA fragments used for targeted gene replacement were amplified by PCR using primers with 70-nt extensions that are homologous to aceA and the resistance cassette-encoding template plasmid pKD4 (CL77: TACCCGCTTATGCTTATACACCCAATATACTGACATCTGCACATGGTGTTAGGCTGGACGTGCTTC, CL78: GGCCTACAGTCAGCAACGGTTGTTGCTTAGAACTGCGA TTCTTACATGCTATATATCTCCTCTTAG). Chemically competent E. coli BW25113 carrying the pKD46 plasmid (Red system) were transformed with the PCR products, and knockouts obtained by recombination were selected via kanamycin resistance. The gene deletion was confirmed by PCR (CL79: AGGAATCGACCATACTGAGCA, CL80: CAGTACATCGAAGCGTGGATC).

4.7 | Photometric assay of isocitrate lyase activity

Bacteria were harvested from agar plates and diluted to a final OD$_{550}$ of 10 in D-PBS (w/o MgCl$_2$, life technologies). After lysis via sonification, unbroken cells and cell debris were removed by centrifugation (10,000 g, 30 min, 4°C). Isocitrate lyase activity was measured using a protocol adapted from Reeves et al. (1971). Briefly, 25 µl bacterial lysate were mixed with 120 µl reaction buffer (10 µl 500 mM Tris–HCl, pH 7.5, 5 µl 100 mM MgCl$_2$, 5 µl 1 mM L-cysteine; 10 µl 100 mM phenylhydrazine–HCl (Sigma), 90 µl ddH$_2$O) in a 96-well plate (clear, flat-bottom). A volume of 5 µl 20 mM DL-isocitric acid trisodium salt hydrate (Acros organics) was added and measurement was immediately started in a pre-warmed plate reader (30°C, Clariostar BMG Labtech). The formation of glyoxylate-phenylhydrazone was recorded by measuring the absorbance at 324 nm every 2.5 min for 30 min. Before each measurement, plates were automatically mixed for 2 s at 500 rpm double orbital shaking. As a blank control, 5 µl ddH$_2$O instead of DL-isocitric acid was added to the respective samples.

4.8 | H. pylori growth curves

Bacteria grown on plates were diluted to an OD$_{550}$ of 0.075 in BB/FCS and subcultured in 96-well plates (clear, flat-bottom). Plates were sealed with a gas permeable membrane (Breathe-Easy® sealing membrane, Diversified Biotech) and incubated at 37°C, 200 rpm in a plate reader (Clariostar, BMG Labtech). CO$_2$ levels were adjusted to 10% using an atmospheric control unit (BMG Labtech). OD$_{550}$ was automatically measured every 5 min until the stationary phase was reached. Alternatively, bacteria were inoculated to an initial OD$_{550}$ of 0.05 in BB/FCS and incubated under microaerobic atmosphere under gentle agitation. At the indicated time points, the OD$_{550}$ was measured manually.

ACKNOWLEDGEMENTS

This work was funded by the German Center for Infection Research (DZIF) Project-ID 8025806810 to RH and by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation)—Project-ID 364653263—TRR 235 to WE. Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

TS, WE, CL, RH, WG and WF designed the study. TS, CL, WE and WF wrote the manuscript. TS, CL and FS performed the experiments. TS, WE, CL, RH, WG and WF designed the study. TS, WE, CL, RH and WG performed the experiments. WE, WF and RH provided the laboratory space and equipment.

ORCID

Wolfgang Goebel https://orcid.org/0000-0003-4851-2083
Wolfgang Fischer https://orcid.org/0000-0002-8683-5089
Wolfgang Eisenreich https://orcid.org/0000-0002-9832-8279

REFERENCES

Aillard, F., Didelot, X., Woltemate, S., Pfaffinger, G., Overmann, J., Bader, R.C. et al (2019) Within-host evolution of Helicobacter pylori shaped by niche-specific adaptation, intragastric migrations and selective sweeps. Nature Communications, 10(1), 2273. https://doi.org/10.1038/s41467-019-10050-1
Atherton, J.C. & Blaser, M.J. (2009) Coadaptation of Helicobacter pylori and humans: Ancient history, modern implications. Journal of Clinical Investigation, 119(9), 2475–2487. https://doi.org/10.1172/JCI38605
Baar, C., Eppinger, M., Raddatz, G., Simon, J., Lanz, C., Klimmek, O. et al (2003) Complete genome sequence and analysis of Wolinella succinogenes. Proceedings of the National Academy of Sciences, 100(20), 11690–11695. https://doi.org/10.1073/pnas.1932838100
Best, A. & Abu Kwaik, Y. (2019) Nutrition and bipartite metabolism of intracellular pathogens. Trends in Microbiology, 27(6), 550–561. https://doi.org/10.1016/j.tim.2018.12.012
Bhargava, P. & Collins, J.J. (2015) Boosting bacterial metabolism to combat antibiotic resistance. Cell Metabolism, 21(2), 154–155. https://doi.org/10.1016/j.cmet.2015.01.012
chemolithoautotroph *Sulfurimonas denitrificans*. *Applied and Environment Microbiology*, 74(4), 1145–1156. https://doi.org/10.1128/AEM.01844-07

St Maurice, M., Cremades, N., Croxen, M.A., Sisson, G., Sancho, J. & Hoffmann, P.S. (2007) Flavodoxin:quinone reductase (FqrB): A redox partner of pyruvate:ferredoxin oxidoreductase that reversibly couples pyruvate oxidation to NADPH production in *Helicobacter pylori* and *Campylobacter jejuni*. *Journal of Bacteriology*, 189(13), 4764–4773. https://doi.org/10.1128/JB.00287-07

Stark, R.M., Suleiman, M.S., Hassan, I.J., Greenman, J. & Millar, M.R. (1997) Amino acid utilisation and deamination of glutamine and asparagine by *Helicobacter pylori*. *Journal of Medical Microbiology*, 46(9), 793–800. https://doi.org/10.1099/00222615-46-9-793

Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Tsugawa, H., Suzuki, H., Nakagawa, I., Nishizawa, T., Saito, Y., Suematsu, Trastoy, R., Manso, T., Fernández-García, L., Blasco, L., Ambroa, A., Pérez del Molino, M.L. et al (2018) Mechanisms of bacterial tolerance and persistence in the gastrointestinal and respiratory environments. *Clinical Microbiology Reviews*, 31(4), https://doi.org/10.1128/CMR.00023-18

Tan, S., Tompkins, L.S. & Amieva, M.R. (2009) *Helicobacter pylori* usurps cell polarity to turn the cell surface into a replicative niche. *PLoS Pathog*, 5(5), e1000407. https://doi.org/10.1371/journal.ppat.1000407

Tannahill, G.M., Curtis, A.M., Adamik, J., Palsson-McDermott, E.M., McGinley, A.F., Goel, G. et al (2013) Succinate is an inflammatory signal that induces IL-1beta through HIF-1alpha. *Nature*, 496(7444), 238–242. https://doi.org/10.1038/nature11986

Tomb, J.-F., White, O., Kerlavage, A.R., Clayton, R.A., Sutton, G.G., Fleischmann, R.D. et al (1997) The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature*, 388(6642), 539–547. https://doi.org/10.1038/41483

Trastoy, R., Manso, T., Fernández-García, L., Blasco, L., Ambroa, A., Pérez del Molino, M.L. et al (2018) Mechanisms of bacterial tolerance and persistence in the gastrointestinal and respiratory environments. *Clinical Microbiology Reviews*, 31(4), https://doi.org/10.1128/CMR.00023-18

Tsugawa, H., Suzuki, H., Nakagawa, I., Nishizawa, T., Saito, Y., Suematsu, M. et al (2008) Alpha-ketoglutarate oxidoreductase, an essential salvage enzyme of energy metabolism, in coccoid form of *Helicobacter pylori*. *Biochemical and Biophysical Research Communications*, 376(1), 46–51. https://doi.org/10.1016/j.bbrc.2008.08.078

Van Acker, H., Sass, A., Bazzini, S., De Roy, K., Udine, C., Messiaen, T. et al (2013) Biofilm-grown *Burkholderia cepacia* complex cells survive antibiotic treatment by avoiding production of reactive oxygen species. *PLoS One*, 8(3), e58943. https://doi.org/10.1371/journal.pone.0058943

Wang, Y., Bojer, M.S., George, S.E., Wang, Z., Jensen, P.R., Wolz, C. et al (2018) Inactivation of TCA cycle enhances *Staphylococcus aureus* persister cell formation in stationary phase. *Scientific Reports*, 8(1), 10849. https://doi.org/10.1038/s41598-018-29123-0

Watanabe, S., Morimoto, D., Fukumori, F., Shinomiya, H., Nishiwaki, H., Kawano-Kawada, M. et al (2012) Amino acid utilisation and deamination of glutamine and asparagine by Helicobacter pylori. *Journal of Medical Microbiology*, 61(9), 1273–1275.

Weiss, M.C., Sousa, F.L., Mrnjavac, N., Neukirchen, S., Roettger, M., Nelson-Sathi, S. et al (2016) The physiology and habitat of the last universal common ancestor. *Nature Microbiology*, 1(9), 16116. https://doi.org/10.1038/nmicrobiol.2016.116

Windham, I.H., Servetas, S.L., Whitmire, J.M., Pletzer, D., Hancock, R.E.W. & Merrell, D.S. (2018) *Helicobacter pylori* biofilm formation is differentially affected by common culture conditions, and proteins play a central role in the biofilm matrix. *Applied and Environment Microbiology*, 84(14), https://doi.org/10.1128/AEM.00391-18

Zampieri, M., Zimmermann, M., Claassen, M. & Sauer, U. (2017) Nontargeted metabolomics reveals the multilevel response to antibiotic perturbations. *Cell Reports*, 19(6), 1214–1228. https://doi.org/10.1016/j.celrep.2017.04.002

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Steiner, T.M., Lettl, C., Schindele, F., Goebel, W., Haas, R., Fischer, W. et al (2021) Substrate usage determines carbon flux via the citrate cycle in *Helicobacter pylori*. *Molecular Microbiology*, 116, 841–860. [https://doi.org/10.1111/mmi.14775](https://doi.org/10.1111/mmi.14775)