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Review

Hypothetical Regulation of Folate Biosynthesis and Strategies for Folate Overproduction in Lactic Acid Bacteria

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ABSTRACT: Folate (vitamin B9) is an essential nutrient for cell metabolism, especially in pregnant women; however, folate deficiency is a major global health issue. To address this issue, folate-rich fermented foods have been used as alternative sources of natural folate. Lactic acid bacteria (LAB), which are commonly involved in food fermentation, can synthesize and excrete folate into the medium, thereby increasing folate levels. However, screening for folate-producing LAB strains is necessary because this ability is highly dependent on the bacterial strain. Some strains of LAB consume folate, and their presence in a fermentation mix can lower the folate levels of the final product. Since microorganisms efficiently regulate folate biosynthesis to meet their growth needs, some strains of folate-producing LAB can deplete folate levels if folate is available in the media. Such folate-efficient producers possess a feedback inhibition mechanism that downregulates folate biosynthesis. Therefore, the application of folate-overproducing strains may be a key strategy for increasing folate levels in media with or without available folate. Many studies have been conducted to screen folate-producing bacteria, but very few have focused on the identification of overproducers. This is probably because of the limited understanding of the regulation of folate biosynthesis in LAB. In this review, we discuss the roles of folate-biosynthetic genes and their contributions to the ability of LAB to synthesize and regulate folate. In addition, we present various hypotheses regarding the regulation of the feedback inhibition mechanism of folate-biosynthetic enzymes and discuss strategies for obtaining folate-overproducing LAB strains.

Keywords: biosynthesis, folate, gene expression regulation, lactic acid bacteria

INTRODUCTION

Folate (vitamin B9) is a conjugated compound composed of a pteridine ring, para-aminobenzoic acid (PABA), and glutamate (Combs, 2008). Folates exist in various forms that are characterized by the oxidation level of the pteridine ring, substituent bound to the N5 and/or N10 position of the tetrahydrofolate (THF) molecule, and number of glutamate residues comprising the polyglutamate tail (Fig. 1). If the pteridine ring is fully oxidized, folate is known as folic acid; if it is partially reduced, it is known as dihydrofolate (DHF); and if it is fully reduced, it is known as THF. Examples of forms with different substituents bound to the N5 and N10 positions of the THF molecule include 5-methyl-THF (5-MTHF), 5-formyl-THF, 10-formyl-THF, 5,10 methylene-THF, 5-formimino-THF, and 5,10-methenyl-THF (Saini et al., 2016; Saubade et al., 2017). Mono-, di-, and triglutamate folates contain one to three glutamate residues in the polyglutamate tail, whereas polyglutamate folates contain more than three glutamate residues (Combs, 2008; Saini et al., 2016).

THF and its derivatives act as cofactors, accepting and donating carbon atoms in one-carbon metabolic reactions, such as DNA synthesis, amino acid synthesis, and the methylation cycle (de Crécy-Lagard et al., 2007; Green and Matthews, 2007; Ohrvik and Witthoft, 2011). DHF is an inactive form of folate that must be reduced to THF during folate biosynthesis in various green plants, bacteria, and yeasts (Green and Matthews, 2007; Wegkamp, 2008). In contrast, folic acid is a synthetic form of folate that is chemically produced in the monoglutamate form and bears no substituents at the N5 and N10 positions (Fig. 1) (Mahara et al., 2019).

Owing to its role as an essential micronutrient, folate requirements in the human body must be adequately fulfilled, particularly during pregnancy (Castaño et al., 2017). Inadequate folate intake during pregnancy can cause various problems, including the risk of miscarriage or still-

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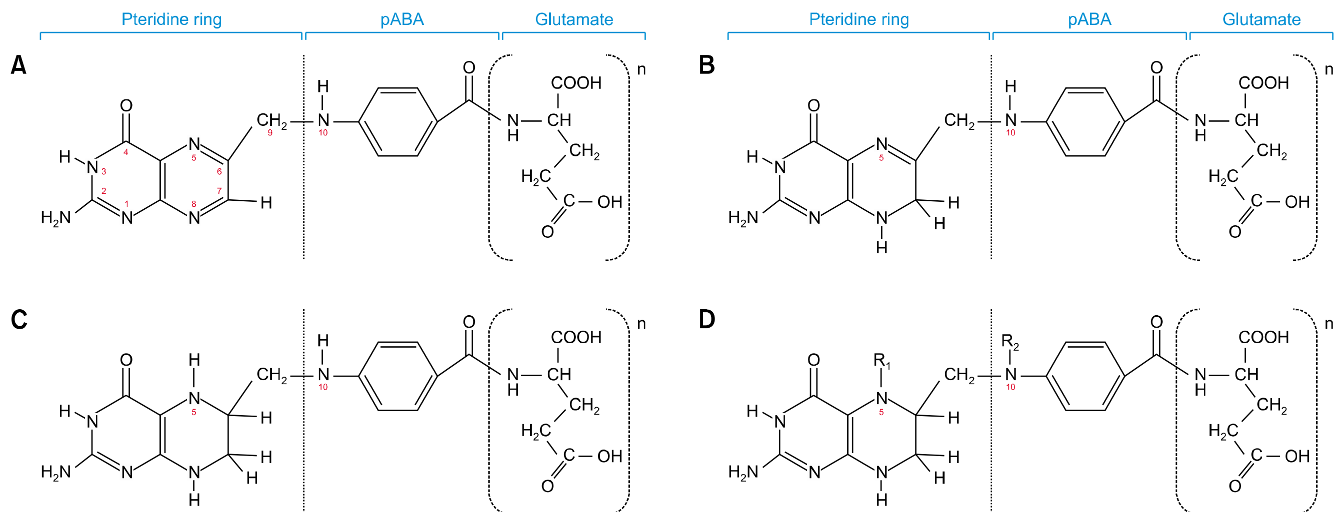

Fig. 1. Chemical structure of (A) folic acid (fully oxidized), (B) dihydrofolate (partially reduced), (C) tetrahydrofolate (THF, completely reduced). (D) THF derivatives with substituents bound to the N5 and/or N10 position.

birth, low birth weight, preeclampsia, prematurity, and neural tube defects (Castaño et al., 2017). However, the use of synthetic folate as a dietary supplement and fortifier has long been avoided, owing to its potential long-term adverse effects on human health (Laiño et al., 2014; Patel and Sobczyńska-Malefora, 2017; Greppi et al., 2017). As an alternative, *in situ* folate fortification via fermentation using folate producing lactic acid bacteria (LAB) has been widely employed to produce safe, efficient, and sustainable biofolate-rich food products (Saubade et al., 2017; Mahara et al., 2019). To obtain high levels of LAB-produced folate, it is necessary to select the appropriate strain and substrate, optimize the fermentation temperature and time, and consider the addition of folate precursors or other compounds (Laiño et al., 2012; Saubade et al., 2017). Certain LAB strains have been reported to be extremely sensitive to folate concentration in the medium, because feedback inhibition can be triggered to inactivate the biosynthetic pathway. As a result, the LAB tend to consume the folate available in the medium (Mahara et al., 2021). This feedback inhibition mechanism acts as a regulator of folate biosynthesis in LAB and therefore efficiently controls their productivity (Scott et al., 2000). Therefore, the application of such LAB is limited to foods that contain no folate to avoid feedback inhibition (Greppi et al., 2017; Mahara et al., 2021).

Previous gene overexpression studies aimed at to generating folate overproducing bacteria have reported the possibility of feedback inhibition via folate-biosynthetic enzymes (Sybesma et al., 2003a; Wegkamp et al., 2007). However, to date, no review has focused on the regulation of folate biosynthesis in LAB or the role of folate-biosynthetic genes in folate-producing and folate-consuming bacteria. This information is expected to lead to an improved understanding of the behavioral patterns of LAB during folate production. Considering previous studies, this review discusses various hypotheses related to the regulation of folate biosynthesis, with the aim of adding to the understanding of how LAB regulate folate synthesis, excretion, and consumption.

FOLATE-PRODUCING LAB

Various genera and species of LAB produce both intracellular and extracellular folate, and this ability is highly strain dependent (Laiño et al., 2012; Greppi et al., 2017). Folate-producing LAB can be isolated at varying levels from various sources, including food sources (raw or fermented) and the digestive tract (Table 1). Strains obtained from the digestive tract can be used as folate producing probiotics, whereas those isolated from fermented foods can be used as starter microbes to manufacture folate-rich food products (Rossi et al., 2011).

Folate synthesis in cells and its excretion into the medium are critical for understanding the potential applications of such systems in food. For example, extracellular folate produced by LAB can increase folate levels in fermented food products. Folate-producing probiotics can be utilized more effectively as producers of extracellular folate because their cells are not lysed in the digestive tract; therefore, they can colonize the colon to continuously provide extracellular folate to the body. In the context of nonprobiotic folate producers, intracellular folate can be produced following cell lysis in the digestive tract, where it can subsequently be absorbed (LeBlanc et al., 2015; Greppi et al., 2017).

Table 1. Folate-producing lactic acid bacteria from various sources

Species (no. of strains tested)	Source	Test medium	No. of folate-producing strains	Folate production (ng/mL)	Reference
Lactobacillus sp. (50)	Traditional Iranian yogurt and doogh	Skim milk medium	50	2.8~66.6	Dana et al., 2010
Streptococcus thermophilus (51)	Artisanal Argentinean yogurt	FACM	32	4.3~76.6	Laiño et al., 2012
Lactobacillus delbrueckii ssp. *bulgaricus* (41)			4	3.6~86.2	
Lactiplantibacillus plantarum (18)	Artisanal Argentinean dairy products	FACM	15	1.4~57.2	Laiño et al., 2014
Lactobacillus acidophilus (8)			2	7.4~37.2	
Limosilactobacillus fermentum (12)			2	0.2~6.9	
Lacticaseibacillus paracasei ssp. *paracasei* (12)			4	9.2~38.7	
Lacticaseibacillus casei ssp. *casei* (3)			0	–	
L. casei (1)			1	1.5	
Lactobacillus amylovorus (1)			1	81.2	
Lactiplantibacillus plantarum (2)	Cereals	FACM	2	30.7~57.3	Salvucci et al., 2016
Limosilactobacillus fermentum (5)			5	5.8~51.1	
Lactobacillus pentosus (3)			3	37.9~61.8	
Levilactobacillus brevis (1)			1	41.3	
Pediococcus acidilactici (6)			6	38.6~55.8	
Pediococcus pentosaceus (1)			1	51.7	
Latilactobacillus sakei (28)	Tocosh (fermented potato porridge)	FACM	28	35~138	Mosso et al., 2018
Lacticaseibacillus casei (9)			4	50~69	
Limosilactobacillus fermentum (1)			1	29	
Levilactobacillus brevis (1)			0	–	
Lactobacillus sp. (2)			1	58	
Streptococcus thermophilus (8)	Fresh milk and several kinds of cheese (cow, goat, and buffalo)	FACM	8	5.06~147.67	Tarrah et al., 2018
Bifidobacterium adolescentis (10)	Human and animals	Folate-free semi-synthetic medium (SM7)	17	0.6~82.0	Pompei et al., 2007
Bifidobacterium animalis (7)					
Bifidobacterium bifidum (6)					
Bifidobacterium breve (15)					
Bifidobacterium catenulatum (1)					
Bifidobacterium cuniculi (3)					
Bifidobacterium dentium (1)					
Bifidobacterium globosom (2)					
Bifidobacterium infantis (5)					
Bifidobacterium lactic (1)					
Bifidobacterium longum (17)					
Bifidobacterium magnum (1)					
Bifidobacterium pseudocatenulatum (3)					
Bifidobacterium suis (1)					
Bifidobacterium thermophilus (1)					
Bifidobacterium sp. (2)					
B. adolescentis (3)	Feces of adults and children	FFM	10	<10,000~92,950	D'Aimmo et al., 2012
B. bifidum (3)					
B. breve (1)					
B. catenulatum (2)					
B. longum (5)					
B. pseudocatenulatum (1)					
B. animalis (3)	Animal feces				

FACM, folic acid casei medium; FFM, folate-free medium.

DE NOVO FOLATE BIOSYNTHESIS PATHWAY AND GENE REGULATION

Folate biosynthesis requires three precursors as the main building blocks: guanosine triphosphate (GTP), PABA, and glutamate. The GTP molecule, which forms the pteridine component of the folate structure, is derived from purine biosynthesis and is an essential molecule synthesized by all LAB (Saubade et al., 2017). Although glutamate can be synthesized through the conversion of α-ketoglutarate from glycolytic intermediates, almost no LAB can synthesize this compound (Lapujade et al., 1998); therefore, glutamate is usually obtained from an external supply (i.e., taken up from the medium) via the salvage pathway (Fig. 2) (de Crécy-Lagard et al., 2007; Iyer and Tomar, 2009). The PABA precursor is derived from chorismate and synthesized via a pathway that is also involved in the aromatic amino acid, glycolysis, pentose phosphate, and shikimate pathways (Rad et al., 2016). Only certain LAB can synthesize this precursor (Rossi et al., 2011); therefore, PABA tends to be obtained from an external supply via the salvage route (Fig. 2) (de Crécy-Lagard et al., 2007).

The *de novo* biosynthesis of folate in bacteria involves the formation of two precursors, namely PABA and DHPPP (6-hydroxymethyl-7,8-dihydropterin pyrophosphate, or pteridine moiety). The combination of this pteridine moiety and PABA with glutamate units generated folate in the form of THF-polyglutamate (Fig. 2) (Wegkamp, 2008; Saubade et al., 2017). This biosynthetic process comprises four pathways: the shikimate pathway (chorismate biosynthesis), PABA biosynthetic pathway, DHPPP biosynthetic pathway, and THF-polyglutamate biosynthetic pathway. Many enzyme-encoding genes involved in these four biosynthetic pathways have been identified, and their presence is associated with the ability of LAB to perform *de novo* folate biosynthesis (de Crécy-Lagard et al., 2007; Rossi et al., 2011; Laiño et al., 2017; Meucci et al., 2018; Laiño et al., 2019).

In the first step of folate biosynthesis, the genes required for the formation of the PABA precursor include *aroF* (3-deoxy-d-arabino-heptulosonate-7-phosphate synthase, DAHPS, EC 2.5.1.54), *aroB* (3-dehydroquinate synthetase, DHQS, EC 4.2.3.4), *aroD* (3-dehydroquinate dehydratase, DHQD, EC 4.2.1.10), *aroE* (shikimate 5-dehydrogenase, SDH, EC 1.1.1.25), *aroK* (shikimate kinase, SK, EC 2.7.1.71), *aroA* (5-*enol*-pyruvylshikimate-3-phosphate synthase, EPSPS, EC 2.5.1.19), and *aroC* (chorismate synthase, CS, EC 4.2.3.5), which are present in the chorismate biosynthetic pathway. In addition, *pabA/B* [aminodeoxychorismate synthase component I (*pabA*) and component II (*pabB*), EC 2.6.1.85] and *pabC* (4-amino-4-deoxychorismate lyase, EC 4.1.3.38), which are present in the PABA biosynthetic pathway, are required (Sybesma,

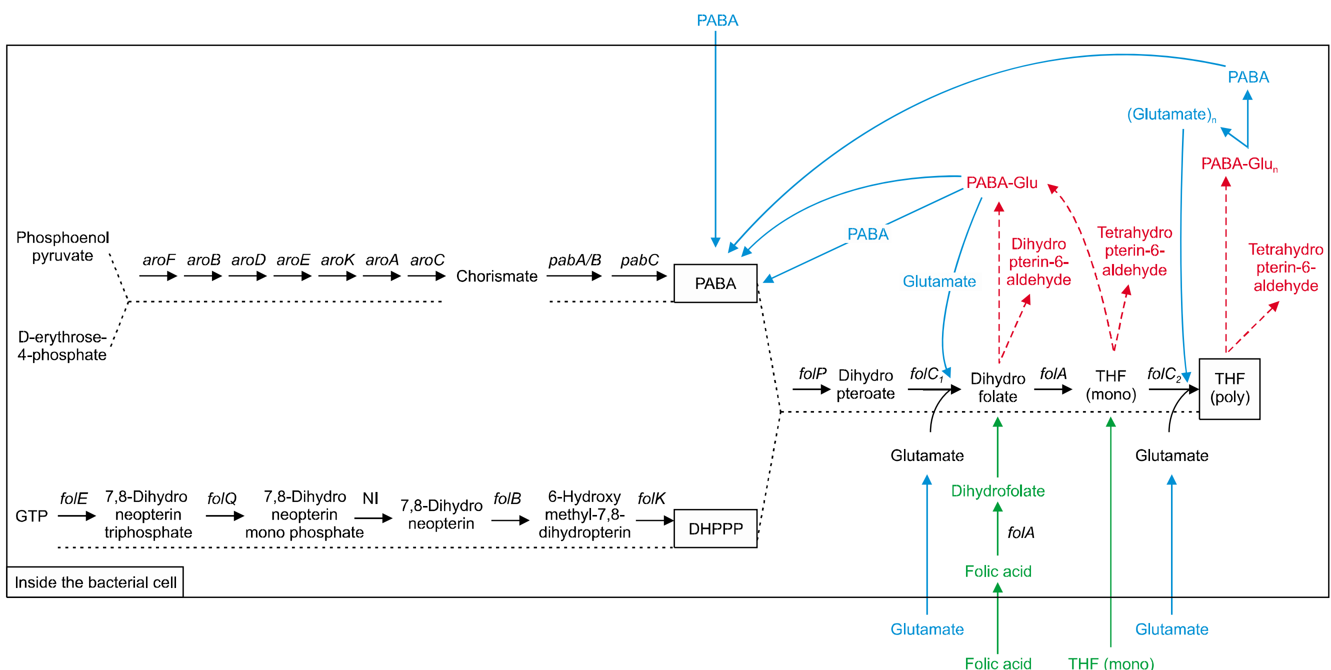

Fig. 2. Folate biosynthesis and salvage pathways in lactic acid bacteria and the genes involved (Orsomando et al., 2006; de Crécy-Lagard et al., 2007; Noiriel et al., 2007; Wegkamp, 2008; Rad et al., 2016). The black arrows indicate the folate biosynthesis pathway, followed by the names of the genes that encode folate biosynthetic enzymes. The blue arrows show the intermediate salvage pathway (PABA and glutamate) obtained from the oxidative breakdown of folate (marked by red dashed arrows) in the cell and taken up from outside the cell (when intermediate compounds are available in the environment or medium). The green arrows indicate intact folate salvage pathways for folic acid and THF (mono). GTP, guanosine triphosphate; PABA, para-aminobenzoic acid; DHPPP, 6-hydroxymethyl-7,8-dihydropterin pyrophosphate; THF (mono), tetrahydrofolate-monoglutamate; THF (poly), tetrahydrofolate-polyglutamate; PABA-Glu, PABA-glutamate; NI, not identified.

2003; Wegkamp, 2008; Rossi et al., 2011). Although the majority of LAB possess all the genes involved in chorismate biosynthesis, only a few possess the two complete genes required for the PABA biosynthetic pathway (Saubade et al., 2017). For example, the genera *Lactococcus* and *Streptococcus* carry out *de novo* synthesis of PABA because they contain all the required genes. In contrast, *Lactobacillus* cannot synthesize PABA in a *de novo* manner because it lacks the genes that encode the PABA biosynthetic enzymes; therefore, *Lactobacillus* is generally only able to perform *de novo* biosynthesis of folate under PABA supplementation (Rossi et al., 2011). Furthermore, the genes required for DHPPP formation include *folE* (GTP cyclohydrolase I, EC 3.5.4.16), *folQ* (dihydroneopterin triphosphate pyrophosphohydrolase, DHNTP, EC 3.6.1.-), *folB* (dihydroneopterin aldolase, EC 4.1.2.25), and *folK* [hydroxymethyl dihydropterin pyrophosphokinase (HPPK), EC 2.7.6.3]. The DHPPP precursor is therefore combined with PABA and glutamate in the THF-polyglutamate biosynthetic pathway, which involves the genes *folP* (dihydropteroate synthase, DHPS, EC 2.5.1.15), $folC_1$ (dihydrofolate synthase, DHFS, EC 6.3.2.12), *folA* (dihydrofolate reductase, DHFR, EC 1.5.1.3), and $folC_2$ (folylpolyglutamate synthase, FPGS, EC 6.3.2.17).

The presence of folate-biosynthetic enzyme-encoding genes has been investigated in various LAB species *in silico* by Rossi et al. (2011). They found that several genes involved in the THF-polyglutamate biosynthetic pathway, including *folC* ($folC_1$/$folC_2$) and *folA* and/or its homologs, were present in all studied LAB species. Meanwhile, genes involved in the DHPPP biosynthetic pathway, including the *folP* gene [involved in dihydropteroate (DHP) formation], were found in only a few species. Bacteria possessing all these genes are considered potential folate producers, regardless of their PABA supplementation requirements.

The DHFR enzyme, encoded by *folA*, is essential for bacterial growth. This enzyme catalyzes the reduction of DHF to THF (the final stage of THF-monoglutamate biosynthesis) and the recycling of DHF produced by thymidylate synthase (encoded by *thyA*) (Levin et al., 2004). In addition, when bacteria require folate from the environment, *folA* is involved in the folate salvage pathway (Fig. 2) (de Crécy-Lagard et al., 2007). Hence, the DHFR enzyme should be found in all folate-requiring bacteria because both folate producers and folate nonproducers require DHFR activity to complete the *de novo* biosynthetic process or salvage pathway (Myllykallio et al., 2003; de Crécy-Lagard et al., 2007). However, the gene encoding this enzyme is not present in all bacteria, possibly due to the presence of another type of DHFR encoded by the *folM* gene (DHFR1) or a flavin-dependent dihydropteroate reductase that fuses with DHFR (known as DHFR2). Although certain bacteria can possess both types of DHFR with the *folA* and *folM* genes, the normal function of DHFR1 (*folM*) probably expands further than simply acting to reduce folate (de Crécy-Lagard et al., 2007).

The *folC* gene is also thought to be present in all folate-requiring bacteria, including both folate producers and nonproducers (Rossi et al., 2011). Folate producers can harbor this gene either in the form of the fusion gene *folC* (encoding the bifunctional enzymes DHFS and FPGS) or two separate genes, $folC_1$ (encoding the monofunctional enzyme DHFS) and $folC_2$ (encoding FPGS). Folate nonproducers that rely on the salvage pathway (Fig. 2) are thought to harbor $folC_2$ (Scott et al., 2000; de Crécy-Lagard et al., 2007). In the salvage pathway, bacteria can only take up folate that contains up to three glutamate residues (i.e., monoglutamate, diglutamate, or triglutamate) owing to the inability of the polyglutamate form (folates containing more than three glutamate residues) to enter cells (Sybesma et al., 2003b). Furthermore, bacteria do not possess γ-glutamyl hydrolase (GGH), which is required to break down folate polyglutamate into monoglutamate (Sybesma et al., 2003c); therefore, they cannot utilize polyglutamate folate from outside the cell. Therefore, folate nonproducers require FPGS enzyme ($folC_2$) to convert THF-monoglutamate into THF-polyglutamate (Fig. 2). Moreover, folate polyglutamate is required for folate retention in bacterial cells (in the cytosol or mitochondria) and for modulating folate-dependent enzymes (as cofactors or inhibitors) that have high affinities for polyglutamate folate (McGuire and Bertino, 1981; Sybesma et al., 2003b; Revuelta et al., 2018).

FOLATE SALVAGE PATHWAY

In the folate-biosynthetic pathway, the availability of the three folate precursors (DHPPP, PABA, and glutamate) is critical for modulating THF synthesis. LAB that lack genes for the biosynthesis of these three substances rely on the salvage pathway, which occurs either within the cell or using compounds that are taken up from outside the cell (i.e., when intermediate compounds are available in the environment or medium). In parasites, such as *Leishmania* and *Trypanosomatids*, folate salvage can occur within the PABA, DHPPP, and THF-polyglutamate biosynthesis pathways (de Crécy-Lagard et al., 2007; Noiriel et al., 2007), whereas in LAB, it only occurs within the PABA and THF-polyglutamate pathways (Fig. 2) (de Crécy-Lagard et al., 2007).

The first step in folate biosynthesis is the formation of pterin compounds (e.g., DHPPP). The pterins are a family of aromatic compounds that function as cofactors for aromatic hydroxylases and are involved in the metabolism of aromatic amino acids, such as phenylalanine, tyrosine, and tryptophan (Scott et al., 2000; Noiriel et al., 2007).

To ensure metabolic activity, pterin must be present in its reduced form (i.e., dihydropterin for folate biosynthesis or tetrahydropterin as a cofactor) (Noiriel et al., 2007). However, pterin is highly unstable and easily oxidized to generate its aromatic form. The reduction of pterin to THF also renders it more susceptible to oxidative breakdown, resulting in the production of oxidized pterin compounds, such as dihydropterin-6-aldehyde, tetrahydropterin-6-aldehyde, PABA-Glu (*p*-aminobenzoyl-glutamate), and PABA polyglutamate (PABA-Glu_n) (Fig. 2) (Orsomando et al., 2006; Noiriel et al., 2007).

Only pterin and folate auxotrophic parasites, such as *Leishmania* and *Trypanosomatids*, are known to possess pteridine reductase 1 (PTR1, EC 1.5.1.33), which catalyzes the reduction of oxidized pterin compounds (i.e., recycling) via the pterin salvage pathway (de Crécy-Lagard et al., 2007; Noiriel et al., 2007). Other organisms including plants, bacteria, and fungi, such as pterin and folate prototrophs, are thought to contain no PTR1 enzymes (Noiriel et al., 2007), and some bacteria, such as *Escherichia coli* and *Saccharomyces cerevisiae*, have been reported to lack PTR1 enzymes (de Crécy-Lagard et al., 2007; Noiriel et al., 2007). As a result, these organisms cannot reuse oxidized pterin compounds in the cell. Noiriel et al. (2007) showed that GTP cyclohydrolase I (*folE*) mutants of *E. coli* and *S. cerevisiae*, which cannot produce dihydropterin, cannot be salvaged by oxidized pterin. This indicates that neither bacterium possesses a pterin salvage pathway as it cannot utilize or reduce oxidized pterin compounds. Despite limited research in this area, the results obtained to date indicate that many folate-producing bacteria do not possess the ability to reduce oxidized pterin compounds, because these bacteria are able to produce pterin and folate in the folate-biosynthetic pathway. Therefore, it has been suggested that folate-producing LAB are unable to reduce oxidized pterin compounds and do not possess a pterin salvage pathway (de Crécy-Lagard et al., 2007; Noiriel et al., 2007). Moreover, LAB possessing incomplete genes in the DHPPP (pterin compounds) biosynthetic pathway are unable to synthesize folate (Mahara et al., 2023). This indicates that these LAB cannot produce folate because they cannot produce or salvage pterins. These observations clearly indicated that LAB (both folate producers and nonproducers) do not possess pterin salvage pathways.

Another component required for folate biosynthesis is PABA, which can be obtained via PABA biosynthetic and salvage pathways (Fig. 2). The PABA salvage pathway begins with the oxidative breakdown of folate, which leads to the generation of PABA monoglutamate (PABA-Glu) or PABA-Glu_n (Orsomando et al., 2006; Noiriel et al., 2007). PABA-Glu can be reused for folate biosynthesis in two ways: through direct processing by the DHPS enzyme in the PABA-Glu form or via hydrolysis into PABA and glutamate (catalyzed by intracellular aminoacyl aminohydrolase or carboxypeptidase G enzymes) and further processing of PABA by DHPS (Fig. 2) (Hussein et al., 1998; Orsomando et al., 2006). Furthermore, PABA can be salvaged from exogenous sources in the environment or through medium supplementation (de Crécy-Lagard et al., 2007). Therefore, folate-producing bacteria that lack the PABA biosynthetic ability can still produce folate from supplemented PABA via the salvage pathway.

Glutamate can also be obtained via a salvage pathway (Orsomando et al., 2006; Wegkamp, 2008), which is derived from the oxidative breakdown products of folate to produce PABA-Glu or PABA-Glu_n. Free monoglutamate (Glu) and polyglutamate (Glu_n) from both products can be reused for folate biosynthesis after hydrolysis by intracellular aminoacyl aminohydrolases or carboxypeptidase G. Free monoglutamate can then be processed further by the DHFS enzyme (encoded by the *folC$_1$* gene) to form DHF, whereas polyglutamate can be processed by the FPGS enzyme (encoded by the *folC$_2$* gene) to promote polyglutamate chain elongation and the formation of THF-polyglutamate (Fig. 2) (Hussein et al., 1998; Orsomando et al., 2006). However, Glu_n cannot be broken down into monoglutamate due to the absence of the GGH enzyme in most bacteria. Although the carboxypeptidases present in some *Bacillus* spp. may possess GGH enzyme activity, further research is required on other bacterial species, particularly LAB (Sybesma et al., 2003c). Additionally, glutamate salvage can occur in the environment or through medium supplementation in the form of monoglutamate (Wegkamp, 2008). After entering the cell, monoglutamate is processed by DHFS and FPGS via the THF-polyglutamate biosynthetic pathway (Fig. 2).

All folate-requiring bacteria can take up intact folate, including folic acid and THF-monoglutamate, via intact folate salvage pathways (Fig. 2) (de Crécy-Lagard et al., 2007). As a synthetic form of folate, folic acid can only be obtained through medium supplementation. In contrast, THF-monoglutamate can be salvaged from natural folate in foods and from bacterial folates in both fermented food and the digestive tract (de Crécy-Lagard et al., 2007; Engevik et al., 2019). Therefore, the presence of folate-producing bacteria in fermented foods and the colon benefits other bacterial populations because folate produced by folate-producing bacteria can be utilized by other folate-consuming bacteria.

FOLATE-EFFICIENT BACTERIA

Most bacteria (known as folate-efficient bacteria) naturally produce metabolites for growth only when needed; thus, their folate production levels are generally not excessive (Pompei et al., 2007; Rossi et al., 2011). In addi-

tion, when the required folate level is reached, the bacteria usually halt their production. Moreover, folate is consumed rather than resynthesized when it is available in the media (Mahara et al., 2021). These bacteria must possess specific metabolic regulations that can efficiently control folate biosynthesis in their cells (Scott et al., 2000). Despite an incomplete understanding of folate-biosynthetic regulation, several studies have identified a possible mechanism for feedback inhibition via the end products of folate-biosynthetic enzymes. Such regulation may include inhibition of DHFR enzyme activity (encoded by *folA*) by THF, inhibition of DHPS enzyme activity (encoded by *folP*) by DHP, and inhibition of HPPK enzyme activity (encoded by *folK*) by DHPPP (Fig. 3).

The activity of the DHFR enzyme in catalyzing the conversion of DHF to THF is possibly inhibited by the final product, THF. Indeed, Sybesma et al. (2003a) reported that increasing folate production in *Lactococcus lactis* by conditioning the overexpression of *folA* (the gene encoding DHFR) resulted in a twofold decrease in folate production. This decline was attributed to feedback inhibition controlling the activity of the DHFR enzyme. The activity of DHPS, which catalyzes the conversion of DHPPP and PABA to DHP, can also be blocked by its end product, DHP, which is a competitive inhibitor of both substrates (Rébeillé et al., 1997; Scott et al., 2000; Kołton et al., 2022). The low inhibitory constant (K_i) of DHP (~5 to 10 μM) for the DHPS enzyme is similar to that of sulfonamide compounds, which are known inhibitors of this enzyme. Additionally, DHP cannot accumulate in the mitochondrial matrix space and is an essential regulatory point in the folate-biosynthetic pathway (Scott et al., 2000).

In addition to DHFR and DHPS, the activity of the HPPK enzyme, which converts 6-hydroxymethyl-7,8-dihydropterin to DHPPP, can also be inhibited by its final product, DHPPP, wherein the rate of DHPPP formation is highly dependent on its utilization by the DHPS enzyme (Mouillon et al., 2002; Meucci et al., 2018). In this context, Laiño et al. (2019) reported a feedback inhibition mechanism for the expression of the *folK* gene (the gene encoding the HPPK enzyme) during fermentation. This gene is overexpressed under maximum folate production conditions and then declines drastically during the extended fermentation time, indicating that when folate production is sufficient for growth, the end product regulates the activities of folate-biosynthetic enzymes, and as a result, folate biosynthesis can be repressed.

The activities of folate-biosynthetic enzymes can be controlled not only by their own end products, but also by the end products of other enzymes involved in the folate-biosynthetic pathway (Fig. 3). DHPS may also be regulated by DHF and THF, which are end products of other enzymes involved in folate biosynthesis. Both products had relatively low K_i values for the DHPS enzymes (~8 to 15 μM), which were slightly higher than the corresponding K_i values of the sulfonamides. The similar range of K_i values for DHF and THF may also indicate that the reduced state of the pterin ring is unrelated to enzyme inhibition (Vinnicombe and Derrick, 1999).

Intermediate one-carbon metabolic products can also regulate folate biosynthesis. For example, the methionine repressor (*metJ*) can control *folE*, a gene encoding GTP cyclohydrolase I, which is not controlled by any kind of feedback inhibition. Therefore, the *folE* gene is extremely sensitive to methionine regulation (Green and Matthews, 2007). Likewise, intracellular homocysteine and methionine pools can regulate the DHFR and FPGS enzymes, wherein methionine acts as a repressor and homocysteine acts as an inducer (Lewandowska et al., 1996; Scott et al., 2000; Kołton et al., 2022). Moreover, since THF is the basis of all one-carbon reactions, one-carbon metabolism products can control the performance of folate-biosynthetic enzymes (Scott et al., 2000).

Currently, little is known about the regulation of the DHNA enzyme encoded by *folB* or the DHNTP enzyme encoded by *folQ*. Although a feedback inhibition mechanism strongly controls the activity of the DHNA enzyme (*folB*) in plants (Chaudhary et al., 2018), the same type of regulation may not exist in bacteria.

FOLATE-OVERPRODUCING BACTERIA

Some folate-producing bacteria can synthesize folate beyond their growth requirements, and are not influenced

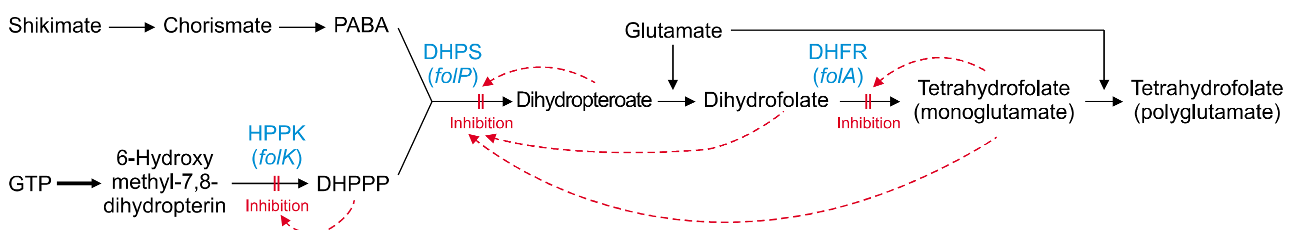

Fig. 3. Summary of the hypothetical feedback inhibition mechanism of folate biosynthesis in lactic acid bacteria. GTP, guanosine triphosphate; PABA, para-aminobenzoic acid; HPPK, hydroxymethyl dihydropterin pyrophosphokinase; DHPPP, 6-hydroxymethyl-7,8-dihydropterin pyrophosphate; DHPS, dihydropteroate synthase; DHFR, dihydrofolate reductase.

by the presence or absence of folate in the medium (Pompei et al., 2007). These folate-overproducing bacteria can be found naturally (Greppi et al., 2017; Albano et al., 2020) or can be produced through genetic engineering (Sybesma et al., 2003a; Wegkamp et al., 2007). Unlike folate-efficient bacteria, folate-overproducing bacteria do not regulate folate biosynthesis in their cells; therefore, the available folate in the medium does not lead to the downregulation of folate. In this context, Pompei et al. (2007) reported that folate levels produced by *Bifidobacterium adolescentis* MB227, *B. adolescentis* MB239, and *Bifidobacterium pseudocatenulatum* MB237 remained constant at high concentrations when grown in media both with and without high-level folate supplementation (50 ng/mL). These bacteria do not control folate biosynthesis; therefore, this process is not affected by the available folate in the medium, which ultimately leads to increased folate levels. This ability to overproduce folate has also been observed in several other LAB species (Table 2).

Despite the wide variety of folate-overproducing LAB species, the discovery of folate overproducers remains a complex task. For example, Mahara et al. (2021) found that three folate-producing LAB (*Lactobacillus plantarum* 4C261, *Lactobacillus fermentum* JK13, and *Lactobacillus rhamnosus* R23) become folate consumers when grown in folate-containing media, indicating that their folate-biosynthetic pathways are tightly regulated by feedback inhibition. Genetically engineered strains must therefore be designed to modify the regulatory mechanism of folate biosynthesis to allow the development of folate overproducing strains (Stanbury et al., 2017). In addition, selection of analog-resistant mutants can be a natural strategy for obtaining folate-overproducing mutants (Wegkamp, 2008; Okoroafor et al., 2019; Zhang et al., 2020). This technique has long been developed as a strain selection procedure for isolating overproducing mutants (Rowlands, 1984; Kumar and Gomes, 2005), and has been successfully used for the production of metabolites, including several types of amino acids (Geeta and Singh, 2000; Kumar et al., 2003; Roy and Mukhopadhyay, 2011) and vitamins (Sybesma et al., 2004; Burgess et al., 2006; Wegkamp, 2008; Capozzi et al., 2011; del Valle et al., 2014; Russo et al., 2014; Zhang et al., 2020). Genetic engineering techniques can also be used to obtain LAB strains with a high capacity for folate overproduction (Sybesma et al., 2003a; Wegkamp et al., 2007; Lu et al., 2019).

FOLATE OVERPRODUCTION BY CHEMICAL ANALOG STRESS RESISTANCE

The stress-resistance method is a classic mutagenesis technique that can trigger spontaneous and directed mutagenesis to obtain mutants with desired phenotypic characteristics (Wegkamp, 2008; Renault, 2010). In this process, microorganisms are exposed to compounds that can inhibit their biosynthetic pathways, such as chemical analogs of targeted metabolites or intermediates (Wegkamp, 2008; Stanbury et al., 2017). Owing to their highly similar structures, these metabolic analogs can compete for binding to biosynthetic enzymes by imitating their control properties, interfering with metabolite biosynthesis, and inhibiting growth (Stanbury et al., 2017). In analog-resistant mutants, this condition triggers excessive production of analogous metabolites, providing additional opportunities for binding to the enzyme. As a result, the enzyme becomes resistant to analog inhibition and loses control of the end-product feedback inhibition (Kumar and Gomes, 2005; Wegkamp, 2008; Stanbury et al., 2017). When regrown in analog-free media, resistant mutants overproduce metabolites without inhibition and excrete them into the medium (Kumar and Gomes, 2005).

The mechanism of analog stress resistance can also be considered in the context of the folate-biosynthetic pathway, which is inhibited by folate analogs and analogs of folate intermediates. For example, Wegkamp (2008) re-

Table 2. Species of folate-overproducing lactic acid bacteria

Species	No. of strains tested	No. of folate-overproducing strains	Folate production (ng/mL)	Reference
Limosilactobacillus fermentum	69	60	0.3~120.9	Greppi et al., 2017
Lactiplantibacillus plantarum	21	17	3.1~110.7	
Lactobacillus paraplantarum	6	5	4.5~16.2	
Pediococcus acidilactici	16	10	0.9~16.5	
Pediococcus pentosaceus	37	0	–	
Lactiplantibacillus plantarum	15	15	5.64~34.41	Albano et al., 2020
Lactococcus lactis	15	1	1.21	
Streptococcus thermophilus	8	1	10.46	
Lactobacillus delbrueckii ssp. *bulgaricus*	6	6	2.86~40	
Lacticaseibacillus casei	7	7	3.33~7.29	
Lacticaseibacillus rhamnosus	7	3	1.28~8.87	
Lacticaseibacillus paracasei	2	2	1.50	

ported that a genetically engineered strain of the folate overproducer *L. plantarum* WCFS1 was resistant to methotrexate inhibition and overproduced folate up to 52-fold in media that lacked folate-dependent metabolites. This finding demonstrates that folate overproduction can trigger resistance to antifolate compounds, such as methotrexate, an analog of DHF. During inhibition, these analogous compounds compete with DHF for binding to DHFR (EC 1.5.1.3); which can inhibit folate biosynthesis and reduce the quantity of cellular folate, thereby significantly reducing the rate of bacterial growth.

Folate overproduction, which can lead to methotrexate resistance, may occur because high intracellular folate production provides additional possibilities to compete with methotrexate for binding to the DHFR enzyme. Thus, the presence of methotrexate did not affect the bacterial growth. However, this resistance mode is more effective if the bacteria are grown in media lacking folate and folate-dependent metabolites, such as purines (inosine, guanine, adenine, and xanthine), pyrimidines (orotic acid, thymidine, and uracil), glycine, methionine, and pantothenate. The folate-dependent metabolites in the media neutralize the growth inhibitory effect of methotrexate; therefore, the resulting bacterial growth is not considered a resistance effect of folate overproduction but is instead due to the presence of folate-dependent metabolites in the media (Harvey, 1973; Wegkamp et al., 2009). Wegkamp (2008) also reported that folate production by *L. plantarum* WCFS1 was three times higher in media lacking folate-dependent metabolites in media containing folate-dependent metabolites. Thus, in the absence of folate-dependent metabolites, the sensitivity of bacteria to methotrexate is increased, and bacterial folate overproduction may result in extreme resistance to methotrexate.

Previous studies have successfully applied this selection method to folate analog-resistant mutants to obtain folate-overproducing mutants (Wegkamp et al., 2009; Zhang et al., 2020). For example, Wegkamp et al. (2009) reported that 1 out of 576 single colonies of *L. plantarum* WCFS1 exhibited natural resistance to methotrexate (≤2.5 mg/mL) and was able to produce 70% more folate than the wild-type. In addition, Zhang et al. (2020) reported that methotrexate-stressed (≤5.0 mg/mL) *L. plantarum* GSLP-7 V could increase folate productivity by up to 2.4 times. Both studies demonstrated that the inhibition of folate biosynthesis via the use of an antifolate compound, such as methotrexate, can trigger excessive folate production by LAB. Okoroafor et al. (2019) also used this approach as an initial screening method for folate-producing bacteria by supplementing media with methotrexate (50 mg/mL), resulting in high folate production levels (40.65 μg/mL for LAB and 52.42 μg/mL for yeast).

However, the generated mutants that exhibit the folate overproduction phenotype frequently revert to their wild-type phenotype after repeated growth in media without the required analogs. In this context, Wegkamp (2008) reported that the high degree of folate production by methotrexate-resistant mutants of *L. plantarum* WCFS1 rapidly decreased in the absence of methotrexate after cultivation for 30 generations. This phenotypic instability, which results in loss of the mutant phenotype, is a common phenomenon. However, Burgess et al. (2006) reported that roseoflavin-resistant mutants of *L. plantarum* NCDO1752 did not lose their riboflavin-overproducing phenotype, even after 60 generations of cultivation without analogs. This may be due to the different modes of roseoflavin resistance, which makes it possible to obtain a mutant with a stable phenotype. Roseoflavin-resistant mutants have been reported to possess mutations in the regulatory region of the rib operon that affect the stability of its terminator structure, thereby altering the regulation of riboflavin biosynthesis. This allows increased transcription of the rib operon, leading to overproduction of stable riboflavin (Sybesma et al., 2004; Burgess et al., 2006). Moreover, mutations in the regulatory region of the operon have been reported to deregulate riboflavin biosynthesis, converting non producer and consumer riboflavin strains into overproducers (Sybesma et al., 2004). Folate overproduction through the stress-resistance method of folate analogs may also exhibit other resistance modes, which should be explored to discover new antifolate compounds that provide mutants with stable folate overproduction phenotypes.

FOLATE OVERPRODUCTION BY GENETIC ENGINEERING

As an alternative strategy for producing folate-overproducing bacteria, microbial genetic engineering can be carried out to strengthen folate-biosynthetic pathways and shift the flux of specific metabolites to target metabolite bioproduction. This can be achieved by inactivating genes, suppressing the expression of unwanted genes, and/or controlling the overexpression of specific genes (Sybesma et al., 2003a; Yang et al., 2020). Reactions that inhibit the accumulation of certain metabolites can be blocked or reduced, whereas reactions that promote the biosynthesis of these metabolites can be amplified (Yang et al., 2020). As outlined in Table 3, genetic modifications have been demonstrated to increase extracellular folate production and alter the distribution and accumulation of intracellular folate. Although the regulation of folate biosynthesis in microorganisms has yet to be fully identified and understood (Wegkamp, 2008; Mahara et al., 2021), several factors that limit folate biosynthesis, such as feedback inhibition of several folate-biosynthetic genes, reversible

Table 3. Folate overproduction in metabolically engineered strains

Microorganisms	Genetic engineering techniques	Results	Reference
Lactococcus lactis MG1363	Overexpression of *folKE*	Increasing the production of extracellular folate 8-fold (±10→±80 ng/mL)	Sybesma et al., 2003a
	Overexpression of *folKE* and *folP*	Increasing the production of extracellular folate 8-fold (±10→±80 ng/mL)	
	Overexpression of *folKE* and *folC*	Increasing the production of extracellular 4-fold (±10→±40 ng/mL) and intracellular folate 3-fold (±50→±150 ng/mL)	
	Overexpression of *folA*	There is no increase in extracellular folate production, and intracellular folate production decreases 2-fold (±75→±35 ng/mL)	
Lactococcus lactis NZ9000	Cloning and expression of the *hgh* gene (*human γ-glutamyl hydrolase*)	Increasing the production of extracellular folate 6-fold (±10→±60 ng/mL)	Sybesma et al., 2003c
Lactococcus lactis NZ9000	Overexpression of PABA genes (*pabA* and *pabBC*)	There is no increase in folate production	Wegkamp et al., 2007
	Overexpression of PABA and folate genes (*folB*, *folP*, *folKE*, *folQ*, *folC*)	Increasing the level of total folate (91.7→2,700 ng/mL)	
Ashbya gossypii ATCC 10895	Overexpression of *AgFOL1* and *AgFOL3*; or overexpression of *AgFOL1* and *AgFOL2*	Increasing the level of total folate up to approximately 2.5-fold	Serrano-Amatriain et al., 2016
	Overexpression of *AgFOL2* and *AgFOL3*	Increasing the level of total folate up to 11-fold	
	Overexpression of *AgFOL1*, *AgFOL2*, and *AgFOL3*	Increasing the level of total folate up to 16-fold (680 ng/mL)	
	Deletion of *AgMET7* (FPGS)	Increasing the level of total folate up to 5.7-fold (292.15 ng/mL), with the increasing proportions of extracellular folate ±30%	
	Repression of *AgRIB1* (GTP cyclohydrolase II)	Increasing the level of total folate up to 4.2-fold	
	Deletion of *ADE12* (adenylosuccinate synthase)	Decreasing the level of total folate	
	Deletion of *ADE12* and *AgMET7*	Increasing the level of total folate up to 11.9-fold	
	Overexpression of *AgFOL1*, *AgFOL2*, and *AgFOL3*; and deletion of *ADE12*	Increasing the level of total folate up to 15-fold (677 ng/mL)	
	Overexpression of *AgFOL1*, *AgFOL2*, and *AgFOL3*; repression of *AgRIB1*; and deletion of *ADE12*	Increasing the level of total folate up to 21-fold (964 ng/mL)	
	Overexpression of *AgFOL1*, *AgFOL2*, and *AgFOL3*; deletion of *AgMET7* and *ADE12*	Increasing the level of total folate up to 51-fold (2,000 ng/mL)	
	Overexpression of *AgFOL2* and *AgFOL3*; deletion of *AgMET7* and *ADE12*; and repression of *AgRIB1*	Increasing the level of total folate up to 146-fold (6,595 ng/mL)	
Bacillus subtilis 168	Deletion of *yitJ*	There is no increase in folate production	Yang et al., 2020
	Deletion of *yitJ*; cloning and overexpression of *metF*	Increasing the production of 5-MTHF 22.3-fold (10.28→229.62 ng/mL)	
	Deletion of *yitJ* and *purU*; and overexpression of *metF*	Increasing the production of 5-MTHF 24.3-fold (10.28→250 ng/mL)	
	Deletion of *yitJ* and *purU*; and overexpression of *metF* and *dfrA*	Increasing the production of 5-MTHF 26.4-fold (10.28→271.64 ng/mL)	
	Deletion of *yitJ* and *purU*; and overexpression of *metF*, *dfrA*, and *folC*	Increasing the production of 5-MTHF 38.9-fold (10.28→±400 ng/mL)	
	Deletion of *yitJ* and *purU*; and overexpression of *metF*, *dfrA*, *folC*, and *pabB*	Increasing the production of 5-MTHF 38.9-fold (10.28→±400 ng/mL)	
	Deletion of *yitJ* and *purU*; and overexpression of *metF*, *dfrA*, *folC*, *pabB*, and *folE*	Increasing the production of 5-MTHF 48.6-fold (10.28→±500 ng/mL)	
	Deletion of *yitJ* and *purU*; and overexpression of *metF*, *dfrA*, *folC*, *pabB*, *folE*, and *yciA*	Increasing the production of 5-MTHF 93.4-fold (10.28→960.27 ng/mL)	
	Deletion of *yitJ* and *purU*; overexpression of *metF*, *dfrA*, *folC*, *pabB*, *folE*, and *yciA*; and repression of *panB3*	Increasing the production of 5-MTHF 124.5-fold (10.28→1,280 ng/mL)	

Table 3. Continued

Microorganisms	Genetic engineering techniques	Results	Reference
	Deletion of *yitJ* and *purU*; overexpression of *metF*, *dfrA*, *folC*, *pabB*, *folE*, and *yciA*; and repression of *thyA1*	Increasing the production of 5-MTHF 135.2-fold (10.28→1,390 ng/mL)	
	Deletion of *yitJ* and *purU*; overexpression of *metF*, *dfrA*, *folC*, *pabB*, *folE*, and *yciA*; and repression of *pheA1*	Increasing the production of 5-MTHF 140-fold (10.28→1,440 ng/mL)	
	Deletion of *yitJ* and *purU*; overexpression of *metF*, *dfrA*, *folC*, *pabB*, *folE*, and *yciA*; and repression of *trpE3*	Increasing the production of 5-MTHF 145-fold (10.28→1,490 ng/mL)	
	Deletion of *yitJ* and *purU*; overexpression of *metF*, *dfrA*, *folC*, *pabB*, *folE*, and *yciA*; and repression of *pheA2*	Increasing the production of 5-MTHF 154-fold (10.28→1,584.34 ng/mL)	
Lactococcus lactis NZ9000	Overexpression of *metF*	Increasing the production of intracellular 5-MTHF up to 18 ng/mL	Lu et al., 2019
	Overexpression of *dfrA*	There is no increase in folate production	
	Overexpression of *thyA*	There is no increase in folate production	
	Overexpression of *glyA*	There is no increase in folate production	
	Overexpression of *folD*	There is no increase in folate production	
	Overexpression of *metF* and *dfrA*	Increasing the production of intracellular 5-MTHF up to ±30 ng/L	
	Overexpression of *metF* and *glyA*	Increasing the production of intracellular 5-MTHF up to ±33 ng/L	
	Overexpression of *metF* and *thyA*	Increasing the production of intracellular 5-MTHF up to ±23 ng/L	
	Overexpression of *metF*, *glyA*, and *folE*	Increasing the production of intracellular 5-MTHF up to ±50 ng/L	
	Overexpression of *metF*, *dfrA*, and *folE*	Increasing the production of intracellular 5-MTHF up to ±73 ng/L	
	Overexpression of *metF*, *dfrA*, *folE*, and the G6PDH gene	Increasing the production of intracellular 5-MTHF up to ±100 ng/L	
	Overexpression of *metF*, *dfrA*, *folE*, the G6PDH gene, and *fau*	Increasing the production of intracellular 5-MTHF up to ±132 ng/L	

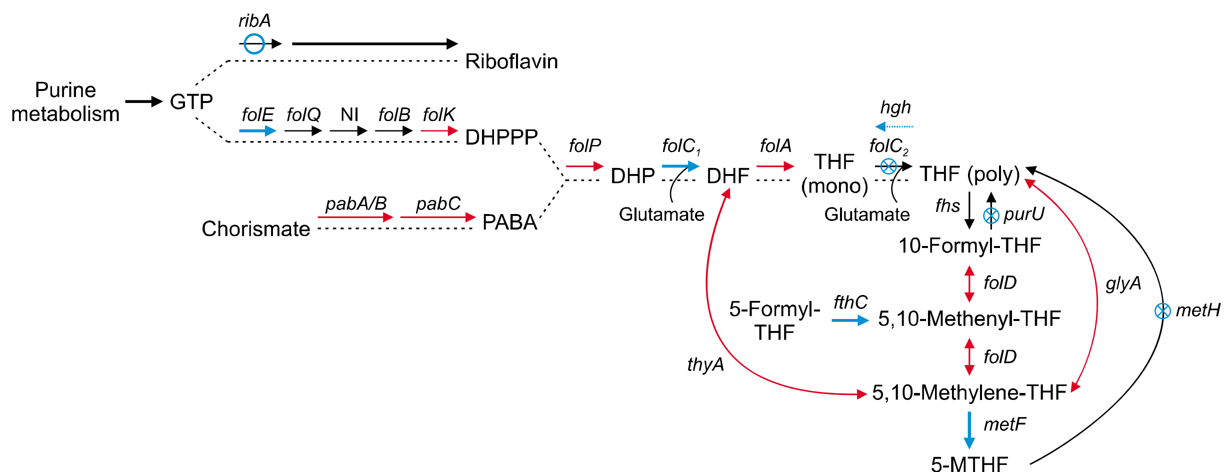

Fig. 4. Schematic of genetic engineering of genes in lactic acid bacteria to develop folate overproducers, based on previous studies. The blue arrows indicate that the gene overexpression technique increased folate productivity, whereas the red arrows indicate no increase. Blue circles represent gene repression and blue crossed circles represent gene deletion; both indicate an increase in folate productivity. GTP, guanosine triphosphate; DHPPP, 6-hydroxymethyl-7,8-dihydropterin pyrophosphate; PABA, para-aminobenzoic acid; DHP, dihydropteroate; DHF, dihydrofolate; THF (mono), tetrahydrofolate-monoglutamate; THF (poly), tetrahydrofolate-polyglutamate.

conversion of the folate form, and complex metabolic pathways, may also influence the application of genetic engineering techniques to construct folate-overproducing strains (Sybesma et al., 2003a; Lu et al., 2019; Yang et al., 2020).

In the first step of the folate-biosynthetic pathway (Fig. 4), which utilizes GTP as a precursor, the *folE* gene (encoding GTP cyclohydrolase I) may be an appropriate tar-

get for gene overexpression to strengthen the folate-biosynthetic pathway. This enzyme has a low turnover rate and is not regulated by feedback inhibition (Sybesma et al., 2003a; Green and Matthews, 2007). Overexpression of *folE* increases extracellular folate production; therefore, the GTP cyclohydrolase I activity may be a limiting step in the folate biosynthesis (Sybesma et al., 2003a; Serrano-Amatriain et al., 2016). In addition, overexpression of the gene encoding the DHFS enzyme (*folC*$_1$ in Fig. 4) increased extracellular folate production, while overexpression of *folC*$_1$ and *folE* resulted in an 11-fold increase in extracellular folate production (Serrano-Amatriain et al., 2016). Despite the unknown regulatory effects of DHFS, its activity is also considered a limiting factor for folate bioproduction (Serrano-Amatriain et al., 2016).

It is also possible that an increase in extracellular folate flux may occur because of the insufficient capacity of the FPGS enzyme to elongate the polyglutamate tail of all extracellular folate produced, because an elongated polyglutamate tail is required for folate retention in cells. When an increase in the extracellular folate flux is followed by an increase in the capacity of FPGS, a shift from extracellular folate flux to intracellular folate accumulation occurs. Under these circumstances, folate retention in the cell increases, leading to an increase in the intracellular folate distribution (Sybesma et al., 2003a). In contrast, when the FPGS enzyme is removed, the production of extracellular folate increases significantly because the produced folate does not possess the polyglutamate tails required for cell retention, and folate is easily excreted from the cell. Deletion of the gene encoding FPGS (*folC*$_2$ in Fig. 4) results in a significant increase in extracellular folate production (Serrano-Amatriain et al., 2016), suggesting that this strategy can also be applied to increase extracellular folate flux.

In contrast to FPGS, the GGH enzyme (encoded by the *hgh* gene), which splits the polyglutamate tail into monoglutamate moieties, enhances the production of monoglutamate folate, which is easily excreted into the medium. However, the bacterial cells do not express this enzyme. The cloning and overexpression of the *hgh* gene (derived from mice or humans) have been carried out in *L. lactis* to increase extracellular folate production by up to 6-fold (Sybesma et al., 2003c).

In addition to being a folate precursor, GTP is also a substrate for the biosynthesis of riboflavin; therefore, the availability of GTP in the cell is reduced for the folate-biosynthetic pathway (Fig. 4). Although deletion of the *ribA* gene (encoding GTP cyclohydrolase II, the first enzyme in the riboflavin pathway) increases extracellular folate flux, it can lead to a deficiency in the *ribA* gene and cause riboflavin auxotrophy. Moreover, riboflavin is an expensive supplement for industrial-scale fermentation; therefore, deletion of the *ribA* gene is economically undesirable. Alternatively, *ribA* gene repression can be used to increase folate flux and maintain the sustainability of riboflavin biosynthesis (Serrano-Amatriain et al., 2016; Yang et al., 2020). Repression of the *ribA* gene has been reported to decrease riboflavin production and significantly increase folate production (Serrano-Amatriain et al., 2016).

The overexpression of folate-biosynthetic genes that regulate feedback inhibition (e.g., *folA*, the gene encoding the DHFR enzyme) can lead to folate downregulation by the end product of the enzyme itself, which does not increase extracellular folate productivity and can even decrease intracellular folate productivity (Sybesma et al., 2003a). Likewise, owing to feedback inhibition regulation, the overexpression of *folK* and *folP* does not increase folate production, although overexpression of the *folKE* fusion gene increases the production of extracellular folate almost 10-fold (Sybesma et al., 2003a). These results suggest that the *folKE* gene, which encodes bifunctional enzymes (HPPK and GTP cyclohydrolase I), may be regulated differently.

The overexpression of *AgFOL1* (encoding the multifunctional enzymes DHNA, DHPS, and HPPK), *AgFOL2* (encoding GTP cyclohydrolase I), and *AgFOL3* (encoding DHFS) has been found to increase folate production up to 16-fold (Serrano-Amatriain et al., 2016). Despite the different regulatory pathways and functions of each gene, multi gene overexpression appears to be a more effective strategy than single-gene overexpression. Although overexpression of the PABA gene cluster (*pabA-pabBC*) (Fig. 4) did not increase folate production, increased folate production was achieved when this gene cluster was combined with the overexpression of other folate-biosynthetic gene clusters such as *folB*, *folKE*, *folP*, *folQ*, and *folC* (Wegkamp et al., 2007).

In recent years, genetic engineering techniques have focused on strategies to increase the biosynthetic flux of 5-MTHF because of its higher bioavailability compared with other forms of folate (Yang et al., 2020; Lu et al., 2019). However, accumulation of 5-MTHF in cells is limited because the conversion of various forms of folate in the 5 MTHF biosynthetic pathway is reversible and involves complex metabolic pathways (Fig. 4) (Lu et al., 2019). Therefore, to shift the metabolic flux to 5-MTHF bioproduction, reactions that inhibit 5-MTHF accumulation must be blocked, whereas those that enhance 5 MTHF biosynthesis must be amplified (Yang et al., 2020). In a study by Lu et al. (2019), the overexpression of several enzyme-encoding genes with reversible activities, such as *thyA* (encoding thymidylate synthase), *glyA* (encoding glycine hydroxymethyltransferase), and *folD* (encoding methylenetetrahydrofolate dehydrogenase) (Fig. 4), did not increase 5-MTHF productivity. However, overexpression of the *metF* gene [encoding the methylenetetrahydrofolate

reductase (MTHFR) enzyme], which catalyzes the conversion of 5,10-methylene-THF to 5-MTHF, resulted in a significant increase in 5-MTHF bioproduction (Yang et al., 2020; Lu et al., 2019). Therefore, the MTHFR enzyme appears to be a key rate-limiting enzyme for 5 MTHF production in the folate conversion pathway. The low expression levels of the *metF* gene in wild-type cells may therefore account for the limited accumulation of 5 MTHF (Lu et al., 2019). Considering these results, *metF* overexpression may be an appropriate strategy to strengthen the 5-MTHF biosynthetic pathway in cells, where the overexpression of *metF* in combination with the overexpression of other genes may confer a large increase in 5-MTHF flux (Table 3).

In the folate conversion pathway, several reactions that limit 5-MTHF accumulation, such as the conversion of 10-formyl-THF to THF and 5-MTHF to THF (Fig. 4), must be blocked to prevent the reversal of the 5-MTHF formation pathway. The deletion of genes that encode the enzymes responsible for catalyzing the reverse reaction should also increase 5-MTHF flux. Indeed, in a study by Yang et al. (2020), which combined the deletion of *yitJ* and *purU* with *metF* overexpression, a large increase in 5-MTHF flux was demonstrated (≤24-fold). A combination of several genetic engineering techniques, such as overexpression, deletion, and gene repression, appears to be the most effective strategy for enhancing folate productivity (Table 3).

In conclusion, folate-producing LAB, including both folate-efficient and -overproducing bacteria, can be used to produce biofolate-rich products. Although various fermentation methods have been found to successfully increase folate production, the regulation of feedback inhibition in folate-efficient bacteria limits their application in foods that do not contain folate. This limitation must be considered when selecting LAB isolates and suitable food types as fermentation substrates to ensure that their application does not decrease folate levels in the final product. The application of folate overproducing bacteria is thought to be advantageous because these organisms can produce folate in quantities that exceed their growth requirements, ultimately increasing the folate concentration in the corresponding food product. Their ability to produce folate in the presence or absence of external folate leads to unlimited application in various foods, thereby rendering the production of biofolate-rich products more facile. Although it is challenging to find this type of bacterium naturally, genetic engineering techniques can be employed for their generation, such as in the case of metabolically engineered generally regarded as safe bacteria, which have been widely used and developed over the last few decades for the bioproduction of specific metabolites.

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The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: LN. Analysis and interpretation: FAM. Data collection: FAM. Writing the article: FAM, LN, HNL, SN. Critical revision of the article: LN, FAM, HNL. Final approval of the article: all authors. Obtained funding: LN. Overall responsibility: FAM, LN, HNL, SN.

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