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Folates biosynthesis by *Streptococcus thermophilus* during growth in milk

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ABSTRACT

The ability of folate-producer strains of *Streptococcus thermophilus* to accumulate folates and the expression of two target genes (*folK* and *folP*), involved in the folate biosynthesis, were studied during milk fermentation. An over-expression of *folK* took place only in the early phase of growth, whereas *folP* was mainly expressed in the mid log-phase of growth and declined thereafter. The accumulation of total folates, which was quantified by a microbiological assay, was strain-dependent. Two major forms of folates, i.e. tetrahydrofolate (THF) and 5-methyl-tetrahydrofolate (5-Met-THF), were identified and quantified by HPLC. With respect to the level accumulated by a weak folate producer (St 383), used as calibrator in the expression experiments and as control in folate quantification in milk, the strains St 563 and St 399 produced 5-Met-THF in amounts significantly higher than THF. The possibility of using selected folate-producer *S. thermophilus* strains as functional cultures for a bio-fortification of dairy products is discussed.

1. Introduction

Folates are compounds having a chemical structure and a nutritional value similar to folic acid. The term "folate" embodies all folic acid derivatives, including the polylglutamate forms of folate naturally present in foods, and folic acid, which is a synthetic folate commonly used as a nutritional supplement for food fortification. Folates are involved in many essential functions of cell metabolism such as DNA replication, repair and methylation, synthesis of nucleotides, amino acids and some vitamins. The deficiency of this important B-group vitamin has been involved in a wide variety of health disorders (Champier et al., 2012; Iyer and Tomar, 2009; LeBlanc et al., 2007). Folates are mainly present in legumes, fruits, vegetables, liver, milk and fermented dairy products. The content of folates in foods of animal origin is generally lower than that observed in vegetables. Although folates are present in various foods, the daily intake of folic acid through the diet can be insufficient to meet the nutrient reference value (NRV, 200 μg for adults) recommended in the European Regulation 1169/2011 (EC, 2011). To address this problem, foods fortified by addition of folic acid have been developed. Unfortunately, food fortification with synthetic molecules may present some downsides. A number of studies seem to raise doubts about the safe use of the chemically synthetized folic acid in foods, addressing the fortification through biological approaches (Iyer and Tomar, 2009). To this regard, some species of lactic acid bacteria (LAB) are able to accumulate folate in milk, suggesting their application as functional cultures in fermented dairy products, thus replacing the artificial fortification with synthetic folic acid (Laiño et al., 2012; Lin and Young, 2000; Rad et al., 2016). The capability to synthesize folates is a property strongly dependent on species, strain and cultivation conditions. Many LAB of dairy interest, such as *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis* subsp. *lactis* and subsp. *cremoris* have demonstrated the ability to accumulate folate during growth in milk (Crittenden et al., 2003; Iyer and Tomar, 2009; Iyer et al., 2010; Laiño et al., 2012; LeBlanc et al., 2011; Lin and Young, 2000; Sybesma et al., 2003).

The proper selection of folate-producing strains, as well as the optimization of the fermentation conditions, are essential prerequisites to increase the folate content in fermented dairy products (Crittenden et al., 2003; Laiño et al., 2013, 2015; Tidona et al.,

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2015). In yogurt fermentation, *S. thermophilus* is the species producing folates, while *Lactobacillus delbrueckii* subsp. *bulgaricus* (as well as many other lactobacilli) usually assimilates folates for growth, reducing therefore their content in the product. Recently, Laiño et al. (2012, 2013) found strains of *L. delbrueckii* subsp. *bulgaricus* capable to synthetize folates in large amounts thus allowing, especially when coupled with folate-producing *S. thermophilus*, to obtain a yoghurt naturally enriched with folates. Therefore, a deeper investigation about the performances and mechanisms of folate accumulation within LAB is of great interest for the application of producer strains in dairy fermentations.

Studies on the expression of folate biosynthesis in *S. thermophilus* are lacking. The *de novo* folate synthesis pathway consists of enzymes involved in the production of the essential parts of the tetrahydrofolate (THF) molecule, principally via a double-branched pathway involving the pterin and pABA branches (Bekaert et al., 2008; Maguire et al., 2014). Three genes (*folB*, *folK*, and *folP*) encoding dihydroneopterin aldolase (DHNA), hydroxymethylpterin pyrophosphokinase (HPPK), and dihydropteroate synthase (DHPS), acting sequentially, are at the core of pterin branch of the folate pathway (Maguire et al., 2014). The present study was aimed at investigating the folate production of *S. thermophilus* in milk. A preliminary genetic screening of 50 strains was followed by a phenotypic selection of folate-producing strains, which were then studied in expression experiments of *folK* and *folP*, two of the key genes involved in the biosynthesis of folates.

2. Materials and methods

2.1. Strains and growth conditions

The 50 strains of *S. thermophilus* used in this study were isolated from traditional Italian dairy products and belonged to the culture collection of the CREA of Lodi (Italy). Pure cultures were stored at −80 °C in M17 broth (Merck, Darmstadt, Germany) added with 20% (v/v) of glycerol. Before use, strains were re-activated in M17 broth supplemented with 1% of lactose at 42 °C for 16 h. The strains selected for the gene expression experiments in milk were preliminarily cultured twice at 42 °C for 18 h in sterile skim milk (SSM; Oxoid, Basingstoke, UK). Enumeration of the *S. thermophilus* viable cells during growth in SSM was carried out on M17 agar, supplemented with 1% of lactose, at 42 °C for 48 h.

2.2. Genetic screening

Genomic DNA was extracted from colonies of the *S. thermophilus* strains grown in M17 agar plates by a chelex-based procedure, according to the method described by Walsh et al. (1991) and stored at −20 °C until use. *In silico* search for the key genes involved in the metabolic pathways of folates was carried out using the genome of *S. thermophilus* CNRZ1066 (Accession number: NC_006449.1) as template. Six genes, i.e. *folP* (dihydropteroate synthase), *folE* (GTP cyclohydrolase I), *folK* (2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase, HPPK), *folQ* (dihydroneopterin triphosphate pyrophosphohydrolase), *pabB* (*para*-aminobenzoate synthetase component I, ADCS) and *pabC* (4-amino-4-deoxychorismate lyase, ADCL) were selected and their presence in the *S. thermophilus* strains was probed by PCR. The primers designed to amplify DNA fragments of the genes *pabC*, *pabB*, *folK-Q*, and *folP-E* are listed in Table 1. The primer sets produced amplicons of 1850 bp (*pabC*), 1666 bp (*pabB*), 790 bp (*folK-Q*), and 1350 bp (*folP-E*). PCR amplifications were performed in 20 μL volumes with 0.5 mM of each primer (Biotez, Berlin, Germany), 2.5 units/100 mL of Taq Gold DNA polymerase (Life Technologies, Monza, Italy), 1.5 mM $MgCl_2$, 20 ng of total DNA, and 200 mM of each dNTP. The amplifications were performed with the following cycling conditions: 10 min at 95 °C; 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; and 72 °C for 7 min. Amplicons were run on 1.0% agarose gels in 1X TAE buffer and gels stained in GelRed™ (Biotium, Hayward, CA, USA) staining solution. One kb plus DNA Ladder (Invitrogen, Milan, Italy) was used as molecular weight marker.

Table 1
Genes and corresponding primer sequences used in this study for the PCR-based genetic screening[a] and for the real-time RT-PCR analysis[b].

Gene	Primers	Sequence (5' - 3')
pabC [a]	For	CGGACAAGCATAATGAATACTCGGAAT
	Rev	GGATTGATAACCGCTTCTATTGCCGA
pabB [a]	For	CCTCAATTCATACAACCCTCTCACA
	Rev	CAGACAAATCTTCACTCACGCCATAA
folK-Q [a]	For	CACTAGTGTCTATT GACTCAAATATTTT
	Rev	CGTTTTTATGGCTATCACGGGGCT
folP-E [a]	For	GAGATAGTCTTAACGACATCACGATT
	Rev	GCAGTCTATCAATTATTGGAAGCTTT
folK [b]	For	CTCGCTTTAGCACTTGACACTCA
	Rev	GCCTGACAAGCACTCAGCAA
	Probe	TTGCCGGCTGAAAG
folP [b]	For	CGCAAGCTGCCTTGGAA
	Rev	CAAAGCCAGGATCAATCCAAA
	Probe	CAGGTGTAGCTAAAGAA
Ldh [b]	For	GTACGTCCAGTAAACATCCCATTG
	Rev	CGGCAGAAGCCTTCATCTTT
	Probe	ACGATGCTGAACAAC

2.3. Phenotypic screening

M17 broth cultures were centrifuged at 7000 rpm for 5 min at 4 °C, the supernatant discarded, and the cells washed three times with sterile osmotic solution (NaCl 0.85%, w/v). The washed cells were inoculated (2%, v/v) in folate-free culture medium FACM (Folic Acid Casei Medium, Difco, USA), and incubated at 42 °C for 18 h. The growth of the strains in FACM was evaluated by measurements of the optical density at 650 nm (OD_{650}).

2.4. RNA isolation

RNA was isolated from milk samples, collected during fermentation at different growth phases, immediately cooled on ice bath and stored at −80 °C until use. After thawing, samples were pretreated by adding an equal volume of 0.5 M EDTA to remove proteins and lipids. Then samples were gently vortexed and centrifuged at 9000 rpm at 4 °C for 10 min. The cell pellets were recovered and washed twice in TE 0.1 M, pH 8.0 by centrifugation at 7500 rpm at 4 °C for 7 min, and finally re-suspended in TES buffer containing 2% of lysozyme (Sigma-Aldrich, Milan, Italy) to favor cell lysis. From lysed cells, total RNA was extracted by TRIzol® Reagent according to supplier's indications (catalog number 15596026, Life Technologies, Monza, Italy).

2.5. Gene expression study

Gene expression of *folK* and *folP* was evaluated by real-time Reverse Transcription-PCR (RT-PCR). In a first trial, which included the 14 strains able to grow and accumulate folates in FACM (see above), the expression of *folK* and *folP* genes was quantified after 6 h of incubation at 42 °C in SSM. A second trial consisted in evaluating *folK* and *folP* expression in two strains, i.e. St 563 and St 399 at pH 6.2, pH 5.5, and pH 5.0, corresponding to different growth phases in milk. Strains St 563 and St 399 were selected taking into account on the similar acidification rates and,