Running title: *Lactobacillus fermentum and vitamin B2*

Riboflavin overproducing strains of *Lactobacillus fermentum* for riboflavin enriched bread

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Abstract

Lactobacillus fermentum isolated from sourdough was able to produce riboflavin. Spontaneous roseoflavin-resistant mutants were obtained by exposing the wild strain (named *L. fermentum* PBCC11) to increasing concentrations of roseoflavin. Fifteen spontaneous roseoflavin-resistant mutants were isolated and the level of vitamin B_2 was quantified by HPLC. Seven mutant strains produced concentrations of vitamin B_2 higher than 1 mg L^{-1}. Interestingly, three mutants were unable to overproduce riboflavin even though they were able to withstand the selective pressure of roseoflavin. Alignment of the *rib* leader region of PBCC11 and its derivatives, showed only point mutations at two neighboring locations of the RFN element. In particular, the highest riboflavin-producing isolates, possess an A to G mutation at position 240, while the lowest riboflavin producer carries a T to A substitution at position 236. No mutations were detected in the derivative strains that did not have an over-producing phenotype.

The best riboflavin overproducing strain, named *L. fermentum* PBCC11.5, and its parental strain were used to fortify bread. The effect of two different periods of fermentation on the riboflavin level was compared. Bread produced using the co-inoculum yeast and *L. fermentum* PBCC11.5 led to approximately two-fold increase of final vitamin B_2 content.

Key words: *Lactobacillus fermentum*, riboflavin overproduction, functional bread, RFN, sourdough

Introduction

Riboflavin is a water-soluble vitamin of the B-group (vitamin B_2) from plants and microbial origin. This molecule is a precursor of the biologically active coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), both involved as electron acceptors in enzymatic reductions needed to fulfill critical metabolic functions (Rivlin and Pinto 2007). Generally, a balanced diet supplies the riboflavin Recommended Daily Allowance (RDA) corresponding to 1.4 mg per day for an adult man (European Food Information Council 2006). The main dietary sources of riboflavin are milk and dairy products, meats, eggs, cereals and dark-green vegetables. An additional contribution to the vitamin B_2 intake is provided by the *in situ* production by the human gut microbiota (LeBlanc et al. 2013). Nutritional deficiency of this vitamin (ariboflavinosis) can cause, among others, skin disorders resembling a pellagra condition, glossitis, cheilosis, angular cheilitis, anemia and brain dysfunction (Rivlin and Pinto 2007). Furthermore, an insufficient intake of riboflavin may impair the metabolism of other vitamins. Ariboflavinosis may not only occur due to dietary inadequacy, but also by the concurrent effects of some drugs, alcohol consumption or an increased requirement due to specific physiological conditions such as pregnancy or breastfeeding, childhood and elderly (Flynn et al. 2003; Powers 2003). A recent study suggests that the current intakes of vitamins from foods lead to at a relatively low risk of low intakes in all age and sex groups across European countries (Mensink et al., 2013). However, the same authors found that vitamin B_2 intakes was more than 5 % below the Lower Reference Nutrient Intake (LRNI), being youth woman and seniors the most affected (Mensink et al., 2013). In accordance, Fabian and coauthors (2012) reported that a considerable percentage (60%) of elderly people was at risk for vitamin B_2 deficiencies. Thus, for certain groups of individuals it may be advisable to supplement their daily intake of riboflavin. In addition, vitamin B_2 seems to be involved in cancer prevention (Wojcieszyńska et al. 2012) and in the pathogenesis of vascular disease by regulation of homocysteine metabolism (Rivlin and Pinto 2007; Tavares et al. 2009). Therefore, riboflavin

supplementation could be an interesting approach to improve the nutraceutical value of specific food.

Development of new foodstuffs beneficial for human health, such as fortified and functional foods, are currently taking increasing proportions of the food industry market. Furthermore, an increasing awareness of the consumers prompts the food-makers to implement alternative environmental friendly solutions in the production processes. In the last years, significant advances has been achieved in the field of the *in situ* bacterial overproduction of the B-group vitamins, including riboflavin (for a comprehensive review see Burgess et al. 2009; Capozzi et al. 2012).

In particular, promising results have been formerly reported for the production at pilot scale of yogurt (Burgess et al. 2006) or pasta and bread (Capozzi et al. 2011) with increased levels of vitamin B_2. In the aforementioned works, riboflavin overproduction was obtained by the metabolism of selected spontaneous roseoflavin resistant strains (Burgess et al. 2006; Capozzi et al. 2011). The molecular determinants for riboflavin biosynthesis are widely distributed between microorganisms and strictly related to the microorganism carrying a complete functional *rib* operon comprising four genes, namely *ribH*, *ribA*, *ribB* and *ribG* (Capozzi et al. 2012). Tolerance to the toxic roseoflavin has been related to mutations in the regulatory region of the *rib* operon resulting in a riboflavin overproducing phenotype (Lee et al. 2009).

Lactic Acid Bacteria (LAB) are important microorganisms in the food industry and have the status of Qualified Presumption of Safety (QPS) (EFSA 2007). Currently only *Lactococcus lactis*, *Leuconostoc mesenteroides* and *Lactobacillus plantarum* among LAB have been investigated for their ability to overproduce riboflavin after roseoflavin exposure (Burgess et al. 2004; 2006; Capozzi et al. 2011). Therefore, new food-grade microbial species should be explored in order to widen the availability of starter cultures targeting specific fields of the food industry.

Cereal-based fermented foods play a significant worldwide role in human nutrition. Although cereal fermentations take the biggest volume among fermented foods, the microbial starter cultures potential has not been yet fully exploited in this sector (Brandt 2013).

In the present study, a strain of *L. fermentum* was isolated from a variety of Italian sourdough and submitted to the selective pressure of roseoflavin. Fifteen derivative isolates were analyzed for their ability to overproduce riboflavin and used in a pilot production of bread. A relationship between point mutations in the *rib* regulatory region and the level of the riboflavin produced was also investigated. To our knowledge, this is the first report proposing the selection of spontaneous roseoflavin resistant mutants from a wild *L. fermentum*.

Materials and Methods

Strains isolation

A sourdough obtained from an Italian variety of durum wheat provided by a local farm was used to isolate typical sourdough LAB species. Briefly, a sample of sourdough was subjected to decimal dilutions in sterile saline solution (8.5 g L^{-1} NaCl) and spread onto plates of MRS agar (Oxoid, Basingstoke, UK) supplemented with 100 mg L^{-1} of cycloheximide (Sigma Aldrich, St Louis, MO). After 48 h of incubation at 30 °C, forty colonies were chosen randomly or based on their different morphology. All the isolates characterized by positive-Gram staining and negative catalase assay were considered as presumptive lactic acid bacteria.

Growth conditions and screening of riboflavin producers LAB

The forty putative LAB strains were routinely grown in MRS broth at 30 °C. The chemically defined medium (CDM) described by Terrade et al. (2009) was used to determine the prototrophy of the strains for riboflavin. The CDM was modified omitting vitamin B_2, increasing its pH to 6.2 and by using only D-glucose as carbon source. For the screening, all the selected isolates were pre-grown in MRS broth to early stationary phase, washed twice, then subcultured three-times in the chemically defined medium as suggested by Terrade and Mira de Orduña (2009). All transfers were carried out at an inoculation rate of 1:1000 (v/v) and incubated at 30 °C during 24 h. Experiments were performed in triplicate. Aliquots from the third subculture were stored at -80 °C in CDM supplemented with 20% of glycerol.

Identification of riboflavin producing LAB

For the identification of the strains at the species level, genomic DNA was extracted by using the Microbial DNA extraction kit (Cabru, Milan, Italy) according to the manufacturer's instructions, followed by PCR amplification of the 16S ribosomal RNA gene using the universal primers BSF8/BSR1541 (Edwars et al. 1989) and High Fidelity Taq polymerase (Roche, Meylan, France). Amplification conditions were 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min 30 s and a final extension at 72 °C for 5 min. PCR fragments were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sent to Primm Biotech (Milano, Italy) for sequencing. Identification of the strains was performed by comparison with sequences available at the NCBI database (GenBank) using the standard nucleotide_nucleotide homology search Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST).

Isolation of roseoflavin-resistant *L. fermentum* mutants

Spontaneous roseoflavin-resistant *L. fermentum* mutants were obtained by exposition of the *L. fermentum* PBCC11 wild strain to increasing concentrations of roseoflavin (Santa Cruz Biotechnology, Santa Cruz, CA), as previously reported (Burgess et al. 2004). Experiments were performed with subsequent inocula in 1 ml of CDM supplemented with roseoflavin from 10 until a maximum of 200 mg L^{-1} with intermediate concentrations of 50 mg L^{-1} and 100 mg L^{-1}. From the culture grown at the higher level of roseoflavin, fifteen single colonies were randomly isolated after spreading onto MRS agar plates and the corresponding stocks were stored at -80 °C in CDM roseoflavin-free supplemented with 20% of glycerol.

Riboflavin overproduction

All the selected derivative roseoflavin-resistant mutant and their parental strain were inoculated in 5 ml of CDM and incubated at 30 °C until cells entered stationary phase (OD_{600} = 2.5). Then, the whole bacterial cultures were used for the riboflavin determination. In addition, the mutants were subcultured consecutively five times, each for 24 hours, and the whole extracts corresponding to

120 h of growth was used to determine the stability of the riboflavin-overproducing phenotype after approximately 100 generations. All the assays were performed in triplicate.

Extraction of riboflavin

Riboflavin whole extracts from both microbial cultures and bread were obtained according to the procedure proposed by Jakobsen (2008). Concisely, 5 grams of samples were supplemented with 25 ml 0.1 M HCl and autoclaved at 121 °C for 30 min. After cooling of the samples, pH was increased up to 4.5 with 4 M sodium acetate and submitted to enzymatic hydrolysis by adding a 5 ml solution containing α-amylase (420 U), papain (12 U), acid phosphatase (22 U) and 0.1% of glutathione (all purchased from Sigma Aldrich). Then, the flasks were located at an ultrasonic bath during 1 h and samples were subsequently diluted up to 50 mL with 0.01 M HCl. All the operations were carried out taking care to appropriately protect the samples from light radiation by covering with aluminum foil. Injection of the samples into the HPLC-system was preceded by a filtration step through a 0.20 µM filter.

Quantitative determination of riboflavin

Chromatographic analyses were performed by a HPLC consisting of a degasser system with nitrogen, a binary pump and a fluorescence detector (Agilent-1100 Series, Palo Alto, CA, USA).

A Zorbax Eclipse Plus C 18 (4,6 x 150 mm, 5 µm i.d.) analytical column with a pre-column Zorbax ODS (4,6 x 12,5 mm, 5 µm i.d.), (Agilent Technologies) was used for the analytical determination of riboflavim. Signals were recorded by a ChemStation computer software (Agilent, Palo Alto, CA, USA).

HPLC analyses were achieved by an isocratic elution at mL min^{-1} using the conditions described by Jakobsen (2008) procedure, with a mobile phase constituted by a methanol:water (35:65 v/v) mixture, freshly prepared every day. The eluate was monitored by a fluorescence detector set at an excitation wavelength of 440 nm and an emission wavelength of 520 nm. Spectra analyses of standard and real samples were performed in order to verify the method selectivity.

Analysis of the RFN element

Chromosomal DNA from *L. fermentum* PBCC11 and its derivative strains was obtained by using the Microbial DNA extraction kit (Cabru, Milan, Italy) according to the manufacturer's instructions. Amount and purity of the extracted DNA were assessed by using a BioTek Eon spectrophotometer (BioTek, VT, USA). *L. fermentum* specific RFN primers were designed on the *Lactobacillus fermentum* IFO 3956 complete genome sequence (GenBank accession number: AP008937.1) to have a length around 20 bases, a GC content of approximately 50% and a Tm around 60 °C. OligoPerfect Designer software (Invitrogen, Carlsbad, CA) was used to select primers sequences. Secondary structures and dimer formation were predicted using Oligo Analyzer 3.0 software (Integrated DNA Technologies, Coralville**,** IA). The primers pair RFNFfm (5'-GTATTGAGCAACCAGCG-3') and RFNR (5'-TGGCCGTCTTTGACTA-3') (Primm Biotech) were used to amplify a 576 bp fragment including the *rib* regulatory region. The amplifications were performed in a 25 µl volume reaction containing 20 ng of DNA, 5 µl of 5x HotStar HiFidelity PCR Buffer (Qiagen), 0.2 nm of each primer and 2.5 U μL^{-1} of HotStar HiFidelity DNA Polymerase (Qiagen). The thermal profile was as follows: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 53 °C for 45 s, 72 °C for 75 s, a final extension at 72 °C for 7 min. Clean-up was performed with a QIAquick PCR purification kit (Qiagen) and quantification and purity of the amplification fragment were determined spectrophotometrically and by visualization on 1.2% agarose gels. Sequencing of the amplicons was executed by Primm Biotech. Multiple sequence alignments of the *rib* operon regulatory region of the *L. fermentum* strains were performed using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo).

Bread-making

Bread was prepared according to the procedure described by Capozzi et al. (2011). Dough was obtained in a pilot plant by mixing 2 kg of wheat flour, 400 mL solution of a commercial strain of *Saccharomyces cerevisiae* (3%), and a 400 mL solution containing sucrose (6%), NaCl (3%) and animal fats (3%). Water was added until the optimum rheological parameters as determined by the farinographic adsorption at 500 Brabender Units. Aliquots of dough, each of 300 g, were inoculated

with the riboflavin over-producing derivative *L. fermentum* PBCC11.5 or its parental strain. Briefly, bacterial cells were harvested at the mid-exponential phase by centrifugation (5000 x g, 5 min), washed twice and added to the dough at a final concentration of 2 x 10^8 CFU mL^{-1}. Control was the dough without *L. fermentum* addition. Fermentation was performed at 30 °C and by testing two different time periods, either 4 h or 16 h. Bread was obtained after cooking in an electric oven at 220-230 °C for 30 minutes. All the experiments were performed in triplicate.

Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA). Pairwise comparison of treatment means was achieved by Tukey's procedure with a significance level of P values of <0.05, using the statistical software Past 3.0.

Culture collection

The strains were deposited to the Spanish Type Culture Collection (CECT) and named CECT 8447 (PBCC11) and CECT 8448 (PBCC11.5) respectively.

Gene bank accession number

The accession number of *Lactobacillus fermentum* 16S RNA is: AB859011

Results

Isolation of *L. fermentum* from an Italian sourdough.

Forty putative LAB strains were randomly isolated from an Apulian (Southern Italy) sourdough. In order to determine the auxotrophy for riboflavin, all the strains were grown in a chemically defined medium (Terrade et al. 2009) lacking vitamin B_2. According to Terrade and Mira de Orduña (2009), we considered riboflavin a non-essential nutritional requirement if the strains were able to growth for three-subsequent subcultures without this vitamin. Nineteen isolates were found to be potentially prototrophic for riboflavin (data not shown) suggesting the presence of a functional *rib* operon in their genome. From the riboflavin producing strains, DNA was extracted and used to amplify a 1500 bp fragment of the 16S rRNA gene. Amplicons sequencing and subsequent *in silico*

analysis allow us to identify three different LAB species: *Lactobacillus plantarum* (fifteen isolates), *Leuconostoc mesenteroides* (three isolates) and *Lactobacillus fermentum* (one isolate).

Selection of spontaneous roseoflavin resistant *L. fermentum* with a stable phenotype.

Since that riboflavin overproducing phenotypes of *L. plantarum* and *Leuconostoc mesenteoides* were already investigated in previous works (Burgess et al. 2006; Capozzi et al. 2011), the only *L. fermentum* strain (named *L. fermentum* PBCC11) was submitted to gradually increasing concentrations of roseoflavin, up to a maximum of 200 mg L^{-1}, according to the previously reported procedure (Burgess et al. 2006). Then, fifteen spontaneous roseoflavin-resistant mutants were isolated, inoculated in the CDM and incubated until the stationary phase (OD_{600} = 2.5). At this phase we found the higher concentration of riboflavin (data not shown). Riboflavin production was qualitatively detectable by the turning of the medium from white to yellow (Fig. 1). The level of vitamin B_2 was quantitative detected by HPLC in the whole cell extracts of the mutants and compared with that produced by the parental strain. Based on the results obtained, we were able to divide the putative riboflavin overproducing phenotypes in higher, lower and unable to overproduce riboflavin (Fig. 2). No riboflavin was detected in the whole extract from the wild *L. fermentum* PBCC11. Twelve spontaneous roseoflavin-resistant mutants were riboflavin-overproducers. In particular, seven strains produced concentrations of vitamin B_2 higher than 1 mg L^{-1}, with *L. fermentum* PBCC11.5 being the best over-producer synthesizing 1203 µg L^{-1} ± 59 (Fig. 2) and, therefore, chosen as representative of this group (higher riboflavin overproducer) for further analysis. By contrast, five strains were able to synthesize only a lower amount ranging from about 150 to 250 µg L^{-1}. *L. fermentum* PBCC11.2 which produced 241 µg L^{-1} ± 38 of riboflavin was chosen as representative of this group (lower riboflavin overproducers) (Fig. 2). Interestingly, three derivatives did not show an over-producing phenotype even though they were able to withstand the selective pressure of roseoflavin (Fig. 2). *L. fermentum* PBCC11.1, was chosen as representative of this group for detailed analysis.

In order to investigate the stability of the riboflavin over-producing phenotype, the vitamin B_2 content in cultures of each mutant was detected after one hundred generations. As reported in Fig. 2, no significant differences were found by comparing the riboflavin produced by *L. fermentum* and its riboflavin overproducing derivatives after successive generations in a roseoflavin free medium. Thus, we confirmed that the riboflavin-overproducing phenotype is stably maintained in the absence of the selection pressure for at least 100 generations.

Genomic analysis of the RFN regulatory region

With the aim to identify mutations potentially associated to the riboflavin overproduction, we amplified the RFN element of *L. fermentum* PBCC11 and its derivative mutants. Primer design was performed on the *rib* leader region sequence selected on the on the *Lactobacillus fermentum* IFO 3956 complete genome sequence (GenBank accession number: AP008937.1). Primer pair, RFNFfm and RFNR, was designed to amplify a 576 bp fragment including the *rib* operon regulatory region of *L. fermentum* (Fig. 3A).

The RFN element was deduced by homology with *L. plantarum* NCDO 1752 (identity of 85.38%) and predicted by RFAM (Burge et al. 2013) (Fig. 3A). Unlike Burgess et al. (2004; 2006) no deletions but only point mutations were found in the RFN region of the investigated mutants (Fig. 3A). In particular, all the highest riboflavin-producer isolates possess an A to G mutation at position 240, while the lower producers *L. fermentum* strains contained an T to A substitution at position 236. In bacteria able to synthesize riboflavin, the transcribed RNF element is supposed to fold into a specific complex secondary structure (Gelfand et al. 1999; Vitreschak et al. 2002). Therefore, the putative folding of the RNF element of the *L. fermentum* strains was predicted with the MFold program (http://mfold.rna.albany.edu, The RNA Institute College of arts and Sciences, University of Albany, NY). The expected structure, comprising five stem-loops and a single root stem, was detected for the wild type strain (Fig. 3B). Interestingly, the point mutation detected in the highest overproducer strains resulted in a marked change of the RNF fold, being composed of only three internal stem-loops in the mutants (Fig. 3C). Thus, this change could be responsible for imparing

the proposed transcriptional attenuation mechanism for Gram-positive bacteria upstream of the *rib* genes (Vitreschak et al. 2002). Moreover, the mutation detected in the PBCC11.2 mutant only resulted in a slight change in the fifth stem loop of RNF (Fig. 3D). This change provoked an increase of the ΔG from -39.5 in the wild type to -40.95 in the mutant, that could slow down transcription favoring an antitermination mechanism and it could be related with the low overexpression levels of riboflavin detected in PBCC11.2. Finally, no mutations were detected in those derivatives unable to over-produce riboflavin.

Overall, the results reported strongly suggest a link between the mutation identified and the ability to overproduce riboflavin.

Riboflavin-enriched bread.

The technological application of the riboflavin over-producing *L. fermentum* isolated in this study was tried on a bread production performed at small-scale level as an example of cereals fermented product. Three different test conditions were adopted. Bread was produced inoculating the dough with only a commercial baking yeast starter or supplemented with PBCC11 (wilde-type strain) or PBCC11.5 (the highest riboflavin overproducer strain). Furthermore, two different fermentation times, (4 and 16 h) were tested, with the aim to optimize the conditions for the riboflavin production. The control bread obtained after 4 hours of fermentation by using only a commercial *Saccharomyces cerevisiae* starter, showed approximately 3.3 μg g^{-1} of riboflavin content (Fig. 4). This levels were also detected when the fermentation was carried out with the commercial yeast and *L. fermentum* PBCC11. The concentration of vitamin B_2 increased up 4.1 μg g^{-1} if bread was fermented with the yeast supplemented with *L. fermentum* PBCC11.5 (Fig. 4). The riboflavin content of bread fermented for 16 h was unchanged when only the baking yeast starter was used. However, a slight increase in riboflavin content was observed when dough was supplemented with both the baking yeast and *L. fermentum* PBCC11. The technological assay at 16 h of fermentation including the co-inoculum yeast and *L. fermentum* PBCC11.5 led to a final vitamin B_2 amount of

6.66 µg g^{-1}, corresponding to an increase of about two-times more than the conventional bread (Fig. 4).

Discussion

Sourdough has played an important role in human nutrition for centuries and is a material of primary importance for the bakery industry as well as an essential contributor for energy intake in developing countries (Guyot 2012). From a microbial perspective, sourdough is a complex ecosystem in which coexist yeasts and several hetero- and homo-fermentative LAB, mainly bacteria belonging to the genus *Lactobacillus*, *Leuconostoc*, *Weissella*, *Pediococcus* (Corsetti and Settani 2007; De Vuyst et al. 2013). Comparative genome analysis revealed a shared occurrence of the riboflavin biosynthesis genes among different LAB (Capozzi et al. 2012) including typical inhabitants of sourdoughs that have not been previously investigated for their riboflavin-overproducing phenotype. Therefore, in this work we randomly isolated forty strains from a local Apulian sourdough and we identified only those able to grow in a chemically defined, riboflavin-free, medium. In recent years, several works have reported on the microbial characterization of traditional Italian wheat sourdoughs in order to establish a relationship between the geographical origin of a sourdough and its microbiota (Corsetti et al. 2001; Ricciardi et al. 2005; Settanni et al. 2005; Catzeddu et al. 2006). Agreeing with our results, *Lactobacillus plantarum* was found to dominate the sourdough microbiota in the traditional Altamura bread with the heterofermentative *Lactobacillus brevis*, *Leuconostoc mesenteroides* and *L. fermentum* representing an important share of the isolated strains (Ricciardi et al. 2005).

The unidentified strains auxotrophic for riboflavin, isolated from the food matrix used in this work and corresponding to approximately 50% of the total, may belong to LAB species other than *L. plantarum, Leuconostoc mesenteroides* and *L. fermentum*. For instance, Minervini et al. (2012) found that *Lactobacillus sanfranciscensis* was the most representative LAB in the microbiotas of 19 Italian sourdoughs. However, the *in silico* analysis of *L. sanfranciscensis* TMW 1.1304, the only strain of this species whose complete genome is available (Vogel et al. 2011), revealed the lack of

the *rib* operon. On the other hand, it is known that prototrophy for riboflavin is a strain-dependent feature. Thus, *L. plantarum* WCFS1 carried an incomplete *rib* operon which is devoid of the entire *ribG* gene and part of the *ribB* gene (Capozzi et al. 2012). 'BLAST' analysis with microbial genomes (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) using *L. fermentum* IFO 3956 gene sequences as query sequences, indicated the occurrence of a functional *rib* operon in all the other nine sequenced *L. fermentum* strains (*Lactobacillus fermentum* CECT 5716, GenBank accession number CP002033.1; *Lactobacillus fermentum* F-6, CP005958.1; *Lactobacillus fermentum* 28-3-CHN, ACQG00000000.1; *Lactobacillus fermentum* 3872, AVCT00000000.1; *Lactobacillus fermentum* ATCC 14931, ACGI00000000.1; *Lactobacillus fermentum* FTDC8312, ASXU00000000.1; *Lactobacillus fermentum* Lf1, AWXS00000000.1; *Lactobacillus fermentum* MTCC 8711, AVAB00000000.1; *Lactobacillus fermentum* NB-22, AYHA00000000.1). However, a genetic screening performed by Turpin et al. (2011) showed that from a collection of sixty-nine *L. fermentum* isolated from African fermented pearl millet slurries, about 10% of the strains harbour a *rib* operon lacking *ribA*, *ribB* or *ribG* genes. Riboflavin biosynthesis is subject to a feedback regulation by the highly conserved RFN element, a riboswitch structure located upstream of the *rib* operon (Gelfand et al. 1999; Abbas and Sibirny 2011). Roseoflavin a natural, toxic analogue of riboflavin, is able to bind the RFN, thus inhibiting transcription of the *rib* operon (Winkler et al. 2002; Ott et al. 2009). Exposure of riboflavin-producing microorganisms to the selective pressure of roseoflavin is a technique that has already been successfully used to select LAB with a riboflavin-overproducing phenotype including *L. plantarum* and *Leuconostoc mesenteroides* (Burgess et al. 2006; Capozzi et al. 2011). Therefore, in this work, *L. fermentum* PBCC11 was subjected to increasing concentrations of roseoflavin and the most promising *L. fermentum* mutant selected was able to produced more than 1100 µg L^{-1}. This amount was higher than the maximum riboflavin synthesized by previously reported derivatives of different LAB species obtained with the same approach. In particular, it was found that roseoflavin resistant *Lactococcus lactis* strains were able to produce approximately 900 µg L^{-1} of vitamin B_2 (Burgess et al. 2004), while this concentration

dropped to a maximum of 600 µg L^{-1} when *Leuconostoc mesenteroides* and *L. plantarum* were used as microbial factories (Burgess et al. 2006; Capozzi et al. 2011). However, a riboflavin production of more than 3 mg L^{-1} was observed for the non-LAB strain *Propionibacterium freudenreichii* (Burgess et al. 2006). Although *P. freudenreichii* enjoys the status of QPS (EFSA 2007), its usage as microbial starter in the food industry is limited, as well as *Lactococcus lactis*, to dairy productions.

The phenotype stability of riboflavin-overproducer LAB is a key issue for their utility in the biofortification of food products. Phenotype instability, resulting in the disappearance of mutant features, is a common phenomenon (Wegkamp et al. 2008). However, spontaneous roseoflavin resistant mutants are reported to hold a stable phenotype for sixty subsequent generations (Burgess et al. 2006; Capozzi et al. 2011). Similarly, we confirmed that the riboflavin production remained unchanged in whole extracts of the all *L. fermentum* mutants after one hundred generations, further supporting their potential as starter for the production of fortified cereal-based foods.

In an attempt to correlate mutations in the RFN regulatory elements with the level of riboflavin produced, the corresponding genomic regions from spontaneous roseoflavin resistant *Bacillus subtilis* (Kil et al. 1992; Coquard et al. 1997), *Lactococcus lactis* (Burgess et al. 2004), *Leuconostoc mesenteroides* and *L. plantarum* (Burgess et al. 2006) strains were sequenced and compared to the respective wild type strains. Mutations in this region were found, suggesting that the overproduction of riboflavin could be caused by the inability of mutated riboswitches to bind FMN and repress gene expression (Lee et al. 2009).

Likewise, the *rib* leader regions of the parental and all derivatives *L. fermentum* PBCC11 were sequenced and analyzed. On the basis of the mutations found in the RFN element we were able to divide the overproducing phenotypes in higher, lower and unable to overproduce riboflavine (Fig. 2). This is consistent with the observation previously reported for *Lactococcus lactis* (Burgess et al. 2004) although the same relationship was unclear by analyzing the RFN element of *L. plantarum* and *Leuconostoc mesenteroides* (Burgess et al. 2006).

The main goal of this work was to isolate *L. fermentum* strains able to enrich *in situ* the riboflavin content of fermented foods of cereal origin. A similar approach was already suggested for the production of different foodstuffs such as yogurt (Burgess et al. 2006) or pasta and bread (Capozzi et al. 2011). To our knowledge this is the first time that *L. fermentum*, a common inhabitant of sourdoughs, was investigated for this purpose. Thus, the best riboflavin overproducing *L. fermentum* PBCC11.5 and its parental strain were used to fortify bread. As expected, the riboflavin level was greater in bread obtained with the over-producing *L. fermentum* strain, while no significant differences were found between breads inoculated with *S. cerevisiae* alone or with the wild *L. fermentum*. Under the latter conditions, the observed amount of riboflavin, 3.3 µg g^{-1}, was attributable to flour and yeasts, both a source of B vitamins. Interestingly, the riboflavin level increased up to about 7 µg g^{-1} in the assay that included an extension of the fermentative phase. This agrees with Capozzi and coauthors (2011) who proposed a 16 h pre-fermentative step as a biotechnological innovation to produce riboflavin enriched pasta. From a quantitative standpoint our results match those recently observed by Capozzi et al. (2011) in bread made by using as microbial starters a mix of commercial baking yeast and riboflavin-overproducing derivatives *L. plantarum* strains. Accordingly, we can state that one hundred grams of the obtained riboflavin-enriched bread containing 0.66 mg of vitamin B_2 are enough to meet approximately 50% of the RDA for an adult human.

L. fermentum is a key microorganism in sourdough technology, contributing to flavour, texture, or health-promoting dough ingredients (De Vuyst et al. 2009). Recently, Weckx et al. (2010a) revealed using a metatranscriptomical approach that mature wheat sourdoughs represent a stabilized ecosystem with *L. plantarum* and *L. fermentum* as the dominating LAB species. Prevalence of both species was also found in sourdoughs from different origin such as rye, spelt (Weckx et al. 2010a; 2010b) and African amylaceous fermented foods (Turpin et al. 2011). The growing interest in the nutraceutical treatment of cereal-based fermented foods is further confirmed by a recent screening,

coupled with a metagenomic analysis, of the genes involved in probiotic and nutritional functions, including riboflavin synthesis (Turpin et al. 2011).

Therefore, we believe that the present work can offer useful insights for the industrial production of sourdough as well as open new perspectives in the field of the functional foods based on cereals matrix.

Acknowledgments

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Legend to figures

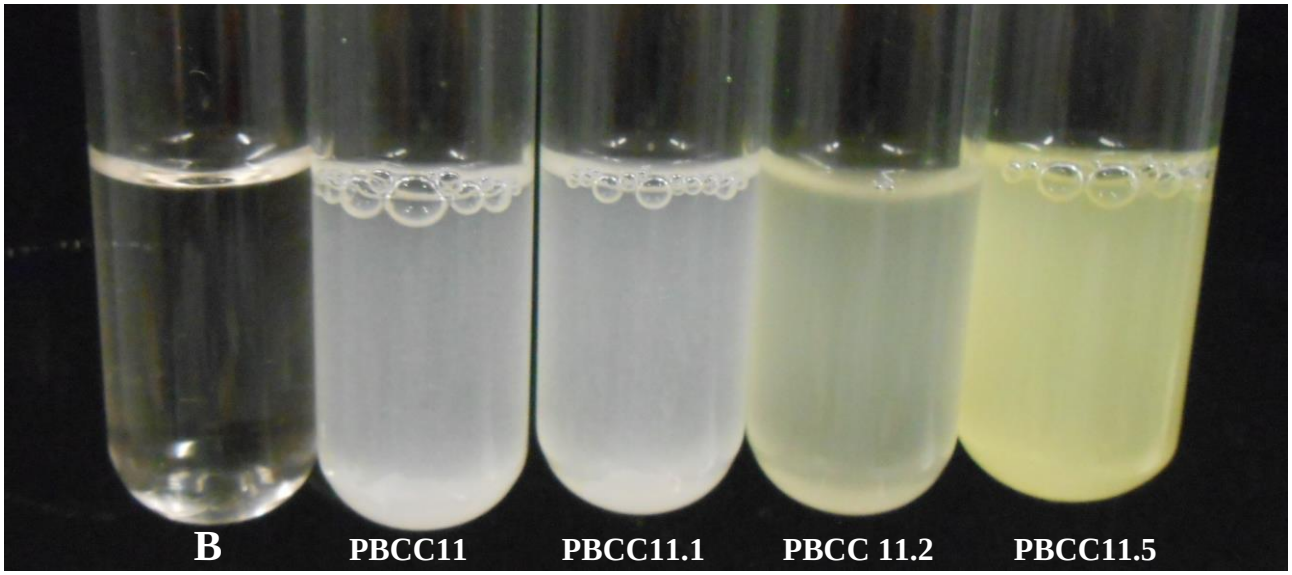
Fig. 1. Example of qualitative detection of riboflavin produced by change of colour from white to yellow in CD riboflavin-free media (B) inoculated with the wild type *L. fermentum* PBCC11 and the derived PBCC11.1, PBCC11.2, PBCC11.5 *L. fermentum* strains.

Fig. 2. Riboflavin produced (µg L^{-1}) by *L. fermentum* PBCC11 and some of roseoflavin resistant derivatives (white bars) chosen on the base of their production ability. The corresponding level after one hundred successive generation (black bars) is reported. Isolates were shown to carry a A to G (*), a T to A (**) or no mutations (***) in the RFN regulator element, when compared to the parental strain. Experiments were performed in triplicate and the standard deviations are indicated.

Fig. 3. (A) Alignment of the *rib* operon regulatory region of *L. fermentum* PBCC11and the two mutants, PBCC11.5 and PBCC11.2, as example of higher and lower riboflavin overproducers, respectively. The mutations are in white font on black background. The predicted -10 and -35 recognition sequences and ribosomal binding site are in bold. The *ribG* start codon and the ribosomal binding site are boxed with dashed and full lines, respectively. The RFN element is indicated by the arrows below the sequence. The predicted folding of the RFN element of PBCC11 (B), PBCC11.5 (C) and PBCC11.2 (D) with the Mfold program is depicted.

Fig. 4. Riboflavin produced (µg g^{-1}) in bread by a commercial bakery *S. cerevisiae* inoculated with or without *L. fermentum* PBCC11 or *L. fermentum* PBCC11.5. Bread was obtained by fermenting the dough 4 h (white bars) or 16 h (black bars). The standard deviations from three experiments are indicated. Different superscript letters indicate statistically significant differences ($P < 0.05$) in the riboflavin content of the samples assessed by one-way Anova test.

Fig. 1



Figure

Figure 2

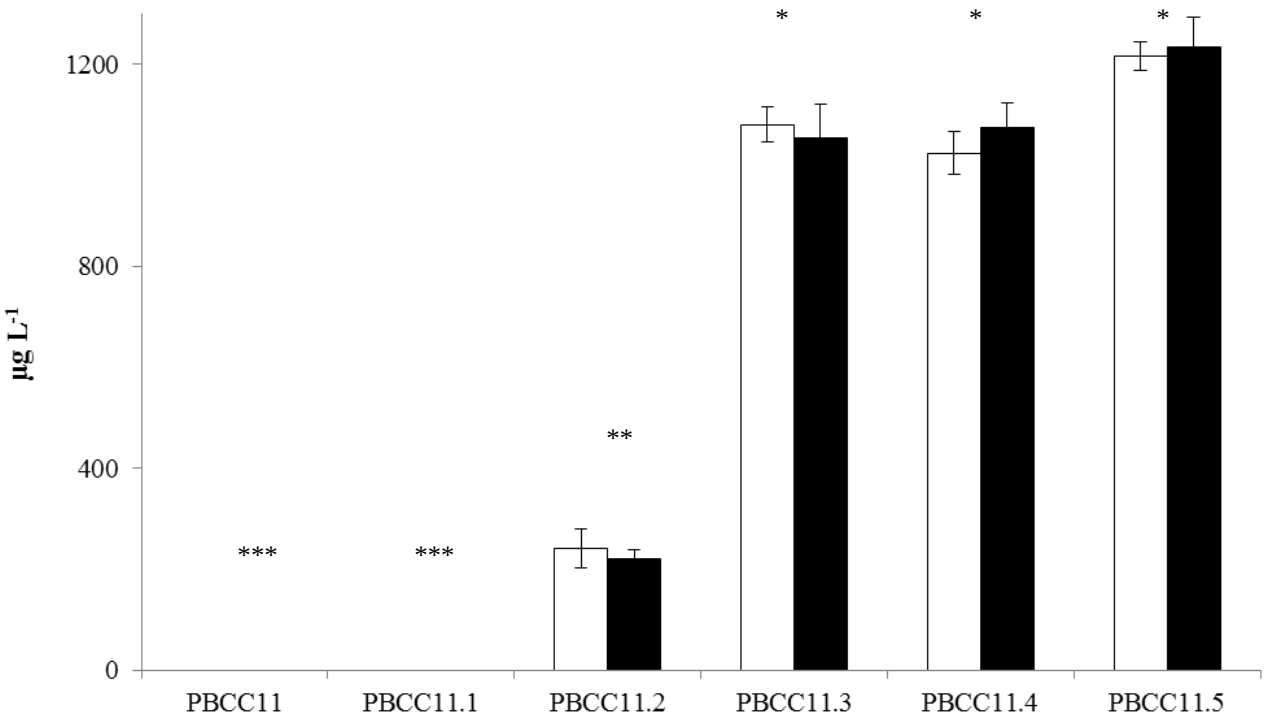
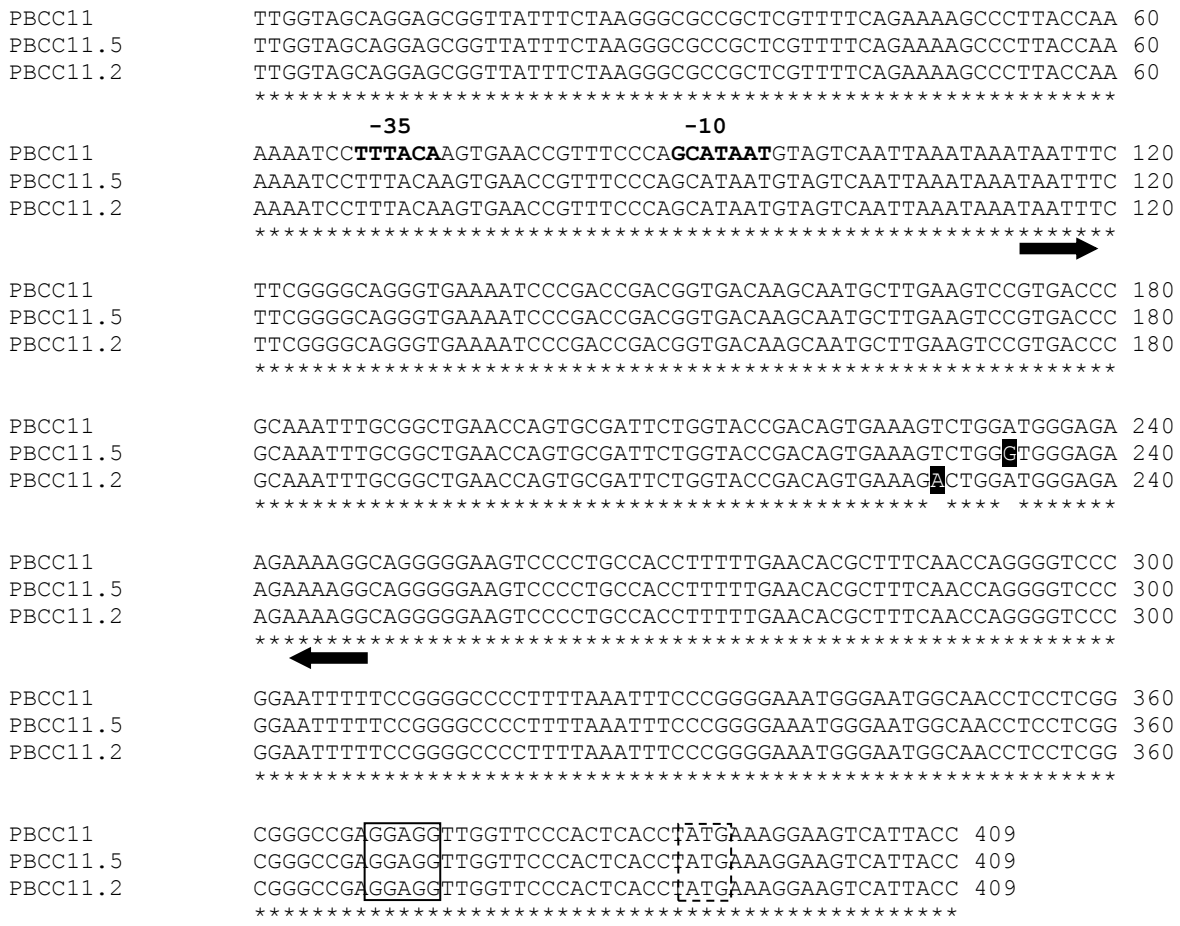


Figure 3

A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PBCC11.2 TTGGTAGCAGGAGCGGTTATTTCTAAGGGCGCCGCTCGTTTTCAGAAAAGCCCTTACCAA 60
-35
-10
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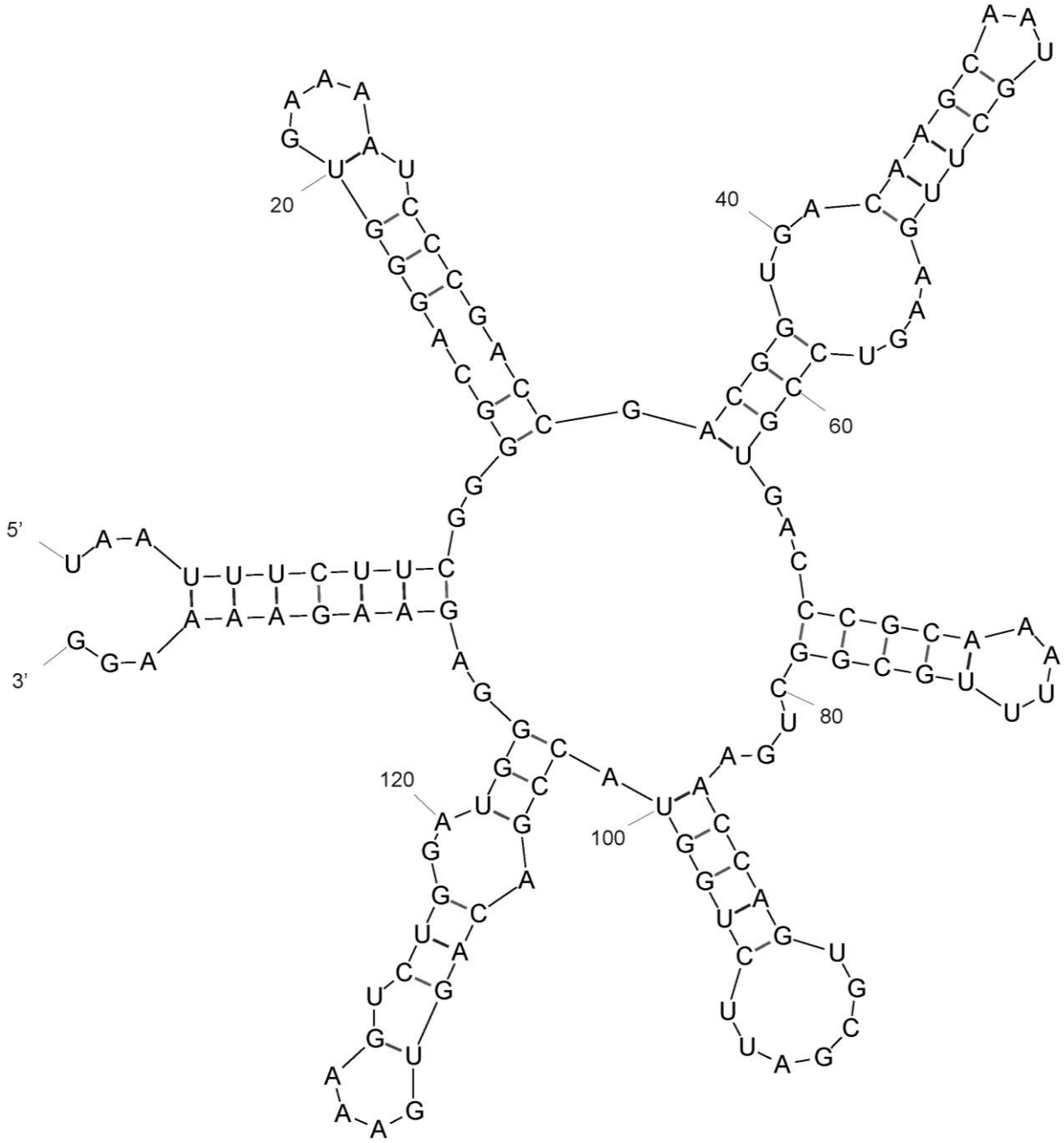
B



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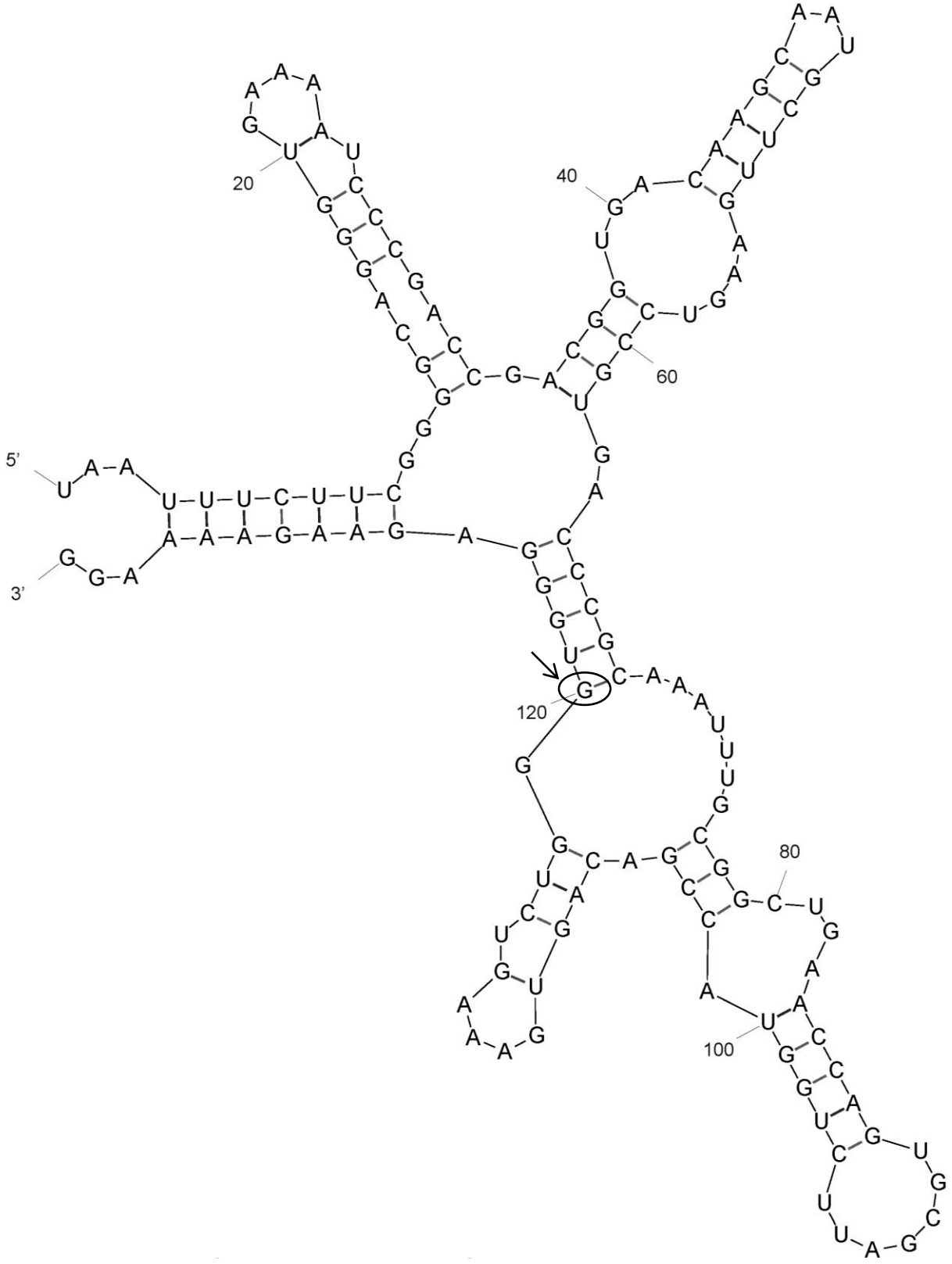

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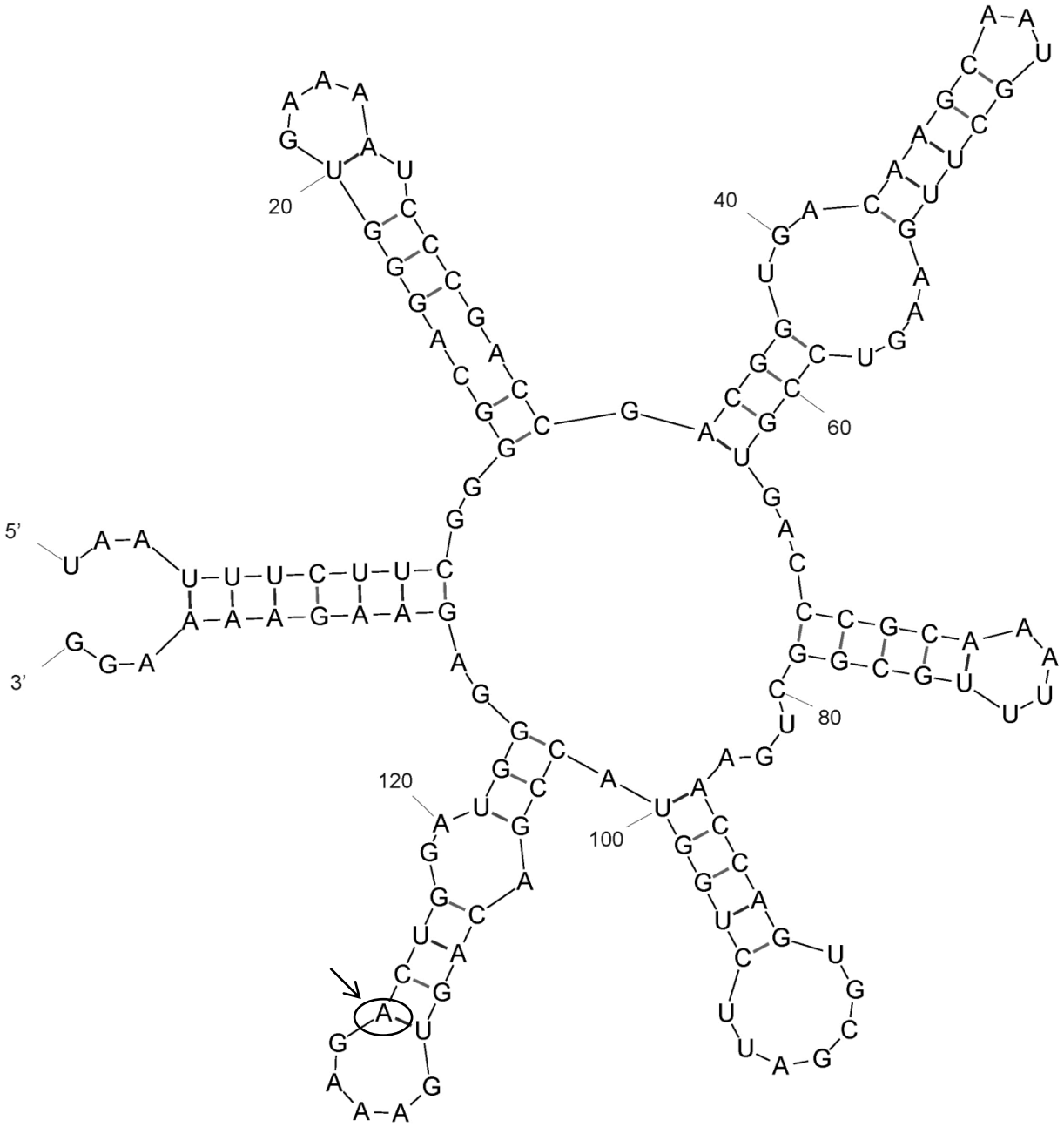

Figure 4