Effect of growth medium composition on the efficiency of non-ribosomal synthesis in bacteria of the genus Bacillus

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Abstract. Bacteria of the genus Bacillus are known for their ability to suppress a wide range of pathogenic microflora through the production of a variety of secondary metabolites, a significant proportion of which are non-ribosomal peptides. The importance of selecting conditions for the most efficient synthesis of non-ribosomal peptides is related to this. The influence of cultivation conditions can be assessed by analyzing the gene expression of non-ribosomal peptide synthetases of target NRPs and studying the mechanisms of its regulation. The aim of this study is to investigate the influence of growth medium composition on the expression of non-ribosomal peptide synthetase genes in Bacillus bacteria. Keywords: *bacillus*, NRPS, non-ribosomal synthesis, growth medium, gene regulation

1 Introduction

Currently, probiotics are effective helpers in combating bacterial infections in animals due to their antagonistic properties. Various non-ribosomal peptides play an important role in exhibiting these properties, showing bactericidal and antifungal activity. For this reason, *in vitro* screening probiotic bacteria for genes encoding synthetase enzymes and selection of nutrient media on which synthesis occurs most efficiently are important aspects in the search for new probiotics.

The optimal composition of the nutrient medium determines the biosynthetic activity of cultured strains and promotes the maximum accumulation of secondary metabolites. Nutrient medium is a factor that needs to be considered for optimizing the production of biologically active substances and studying metabolic processes in microorganisms. Different components of nutrient media influence specific metabolic pathways and synthesis processes. Often nutrient media can lead to the expression of certain genes, which can promote the production of specific bioactive substances. The aim of this study is to investigate the influence of growth medium composition on the expression of non-ribosomal peptide synthetase genes in *Bacillus* genus bacteria.

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2 Probiotics

Probiotics are microorganisms and substances of microbial origin that have a beneficial effect on the physiological, biochemical and immune responses of the host organism by stabilising and optimising the function of the intestinal microbiota [3]. Probiotics have a complex effect: they exert antagonistic activity against pathogenic and opportunistic microorganisms through the production of antibiotics [4], bacteriocins, lysozyme, organic acids (lactic, acetic, succinic, formic), hydrogen peroxide, as well as through competition for habitats and nutrients. Probiotics participate in digestion by synthesising hydrolytic enzymes - analogues of digestive enzymes of the macroorganism; they produce amino acids, vitamins and other biologically active substances consumed by the macroorganism. They have an immunomodulatory effect, destroy toxins and allergens, and reduce the level of cholesterol in the blood. By inhibiting the growth of undesirable microorganisms, probiotics create conditions for the development of a normal intestinal microflora, which plays an extremely important and multifunctional role in the vital activity of the host organism: it provides resistance to colonisation, performs digestive, synthetic, immunomodulatory and detoxifying functions [5]. The intestinal microbiota is considered an independent "organ" that covers the intestinal wall with a biofilm that prevents the introduction of foreign microorganisms and plays an important role in intestinal homeostasis [6].

3 Nonribosomal peptides and their regulation

Non-ribosomal peptides are secondary metabolites of bacteria - short polypeptide chains (2-48 amino acids) that are synthesised without the involvement of ribosomes and the translation of genetic information on them [7]. A characteristic of non-ribosomal peptides is the inclusion of D-amino acids in their composition, which is atypical for eukaryotes, but L-isoforms of amino acids are also present. In addition to prothenogenic amino acids, NRP also includes modified amino acids (methylated, glycosylated and hydroxylated) and non-proteinogenic amino acids [8]. They are formed as a result of non-ribosomal synthesis using enzymes from the family of non-ribosomal peptide synthetases (NRPS) [9].

NRPS are represented by large multi-enzyme systems consisting of modular proteins [9]. The individual NRPS modules are linked by short peptides (peptides of communication interaction, PVCs), each module being responsible for the incorporation of one amino acid. In a bacterial cell, non-ribosomal peptide synthetases are located along the cytoplasmic membrane [10]. The genes of these synthases are characterised by a cluster structure; the synthases themselves are organised into modules containing several domains. The composition of the NRPS complex includes an initiation module, several elongation modules and a termination module. Each module contains catalytic domains, including adenylation, thiolisation, condensation and thioesterase domains. Modifying domains such as epimerisation and methyltransferase domains may also be present [11]. Special attention should be paid to the non-ribosomal peptides investigated in this study.

3.1 Fengycin

Fengycin is a biologically active cyclic lipopeptide containing β-hydroxy acid with a side chain of about 18 carbon atoms attached at the N-terminus. Fengycin is produced by the strain under investigation of Bacillus - R5 (Bacillus velezensis). The NRP consists of ten amino acids, eight of which are included in a cyclic structure. The synthesis of fengycin is controlled by the fen operon, which contains the following genes: fenC, fenD, fenE, fenA and fenB. The synthesis is non-ribosomal, using five fengycin synthetases: FenC, FenD,

FenE, FenA and FenB. Two amino acid activation modules encoded by fenC (the first operon gene) activate the first two amino acids of the fengycin molecule. The final amino acid activation module is encoded by the fenB gene [12, 13].

Fengycin, like other biosurfactants, has surface-active properties that allow it to interact with bacterial membranes and alter their permeability [14]. Its activity against filamentous fungi has been demonstrated [15], as well as antibacterial activity against a wide range of gram-positive and gram-negative bacteria, including pathogens of skin, respiratory, urinary and gastrointestinal infections [16].

3.2 Iturin

Iturin is a cyclic lipopeptide, consisting of seven alpha-amino acid: D-tyrosine, 2 D-asparagine, L-proline, L-serine, L-asparagine, L-glutamine. A peculiarity of this NRP is the inclusion of a lipophilic β-amino acid in its composition [17]. It was found in the R5 strain (Bacillus velezensis). The iturin operon contains four open reading frames, namely ituA, ituB, ituC and ituD. ituA, ituB and ituC contain amino acid activation modules encoding a peptide, and ituD is responsible for encoding malonyl-CoA transacylase, which regulates fatty acid biosynthesis [18]. Iturin has been shown to have antifungal activity and this NRP also disrupts the cell wall [19].

3.3 Bacilysin

Bacilysin is a biologically active dipeptide consisting of the proteinogenic amino acid L-alanine and the non-proteinogenic amino acid L-anticapsine. The main genes for the synthesis of bacilysin are in the ywfA-bacABCDE-ywfG-ywfH cluster. The bacA, bacB and bacC genes are responsible for the modification of prefenic acid to anticapsin, while the bacD gene encodes an L-amino acid ligase that catalyses the amide bond between L-alanine and L-anticapsin residues [20]. Among the strains of the Bacillus investigated R4 (Bacillus subtilis) and R5 (Bacillus velezensis) are capable of synthesising bacilysin.

Research have demonstrated the antibacterial effect of bacilysin: anticapsin inhibits the synthesis of glucosamine, which provokes a malfunction in the synthesis of the bacterial cell wall and ultimately in the growth of the bacteria [21].

3.4 Bacillin

Bacillin is a polyene polyketide obtained by hybrid synthesis using type I PCS and the NRPS operon (baeJ, baeL, baeM, baeN and baeR). Bacillin is a linear molecule consisting of a strongly conjugated hexaene linked to alpha-hydroxycarboxylic acid and a conjugated triene by two amide bonds [22]. This polyketide was found in three strains of the Bacillus: R1 (Bacillus subtilis), R4 (Bacillus subtilis) and R5 (Bacillus velezensis).

Bacillin has antibacterial activity associated with translation arrest [23] and stimulates biofilm formation in the producing strain [24].

3.5 abrB

The abrB gene is responsible for the negative regulation of the synthesis of the studied NRPS (fengycin, iturin, bacilysin). It is a repressor and activator of gene transcription, is expressed during the transition state between vegetative growth and the beginning of the stationary phase and sporulation [25]. 39 operons have been identified which are influenced by abrB; it regulates the production of extracellular degrading enzymes and

amino acid metabolism, membrane bioenergetics and cell wall functions, and biofilm formation [26].

4 Materials and Methods

The research was conducted on strains of Bacillus, namely R1 - Bacillus subtilis, R4 - Bacillus subtilis and R5 - Bacillus velezensis (intralaboratory nomenclature was used). The strains were cultured on four variants of liquid nutrient media: LB (as control), Landi-M (modified Landi medium), soy medium and intestinal media at 25 degrees.

Subsequently, total RNA was isolated from bacterial cultures.

For RNA isolation, we used ExtractRNA reagent (Evrogen) and MMLV RT kit (Evrogen) to generate complementary DNA on the isolated RNA matrix according to the manufacturer's instructions. The qPCRmix-HS SYBR kit (Evrogen) was used for the PCR reaction. The -2deltaCT method and the deltaCT comparison method between control and experiment for regulator genes were used to assess the expression since, in most cases, their value. Statistical analysis was performed in Microsoft Excel using Student's t-test ($P < 0.05$). Analysis of the applicability of the Student's t-test was performed using the Excel add-in AtteStat, in particular, the normality of the data was checked by the Shapiro-Wilk test.

Gene expression was studied using quantitative real-time PCR on a BioRad CFX 96 amplifier.

Amplification was performed according to the following program:

1.Pre-denaturation of DNA at 95 °C for 5 min

2.Denaturation at 95°C for 30 sec.

3.annealing of primers at 51 to 58,5°C for 30 sec

4.Elongation at 70 °C for 40 sec

Steps 2 to 4 were repeated 45 times

5.Analysis of melting curves in the temperature range from 65 to 95°C with an increase in temperature by 0.5°C every 5 seconds.

The gene responsible for the synthesis of the first transcription factor in Bacillus bacteria (sigF) were used as references.

To study the regulation of NRP (nonribosomal peptide) synthesis, we selected primers for the NRPS (nonribosomal peptide synthetases) genes of interest. Thus, we studied the expression of the genes: fenC - accountable for the synthesis of fengycin synthetase subunit C, ituD - accountable for the synthesis of malonyl-CoA transacylase, which regulates the biosynthesis of fatty acids, bacD - encoding the L-amino acid ligase, baeS - accountable for the synthesis of bacillin synthetase subunit S, abrB - responsible for the synthesis of the transcription regulator AbrB.

5 Results and discussion

The changes in gene expression level of fengycin synthetase genes were analyzed, as this is the most common NRP among these strains, as well as bacillin. In addition, several sets of low-specific primers for the most typical NRPs were also selected in order to detect previously unidentified substances. For this purpose, primers for iturin-like and bacilysin-like substances were selected. The expression of iturin synthetase and bacilysin synthetase was studied.

The results are presented in figures 1-5.

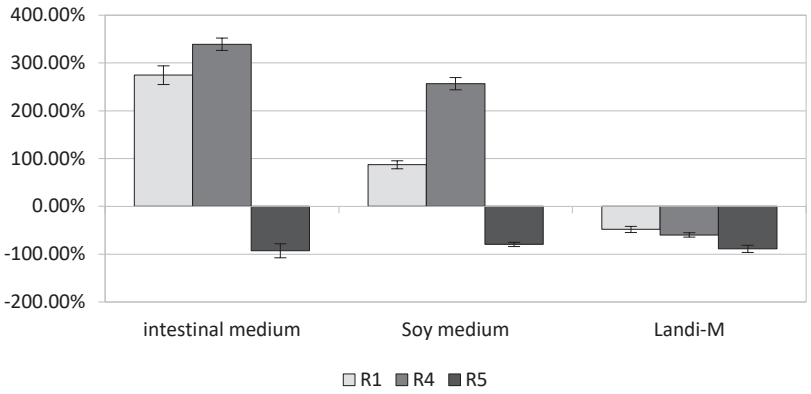

Fig. 1. Expression change of abrB regulator gene expression on different media.

The expression of the gene of the regulator of non-ribosomal synthesis abrB depending on the medium composition was also investigated; the sigma factor gene was used as a reference gene. Four variants of nutrient media were used: LB (as control), modyfied Landi (Landi-M), soy and intestinal media.

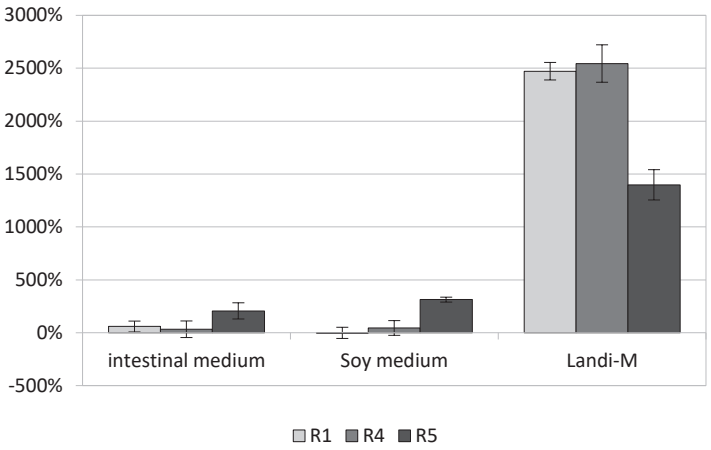

Fig. 2. Changes in bacillin synthetase gene expression on different media.

R5 shows an increase in bacillin synthetase expression level on all media. The expression level of bacillin synthetase gene does not change statistically significantly on intestinal and soy medium for strains R1 and R4. Bacillin synthetase expression increases significantly on Landi-M medium, 25-fold for strains R1, R4. And 14-fold for R5.

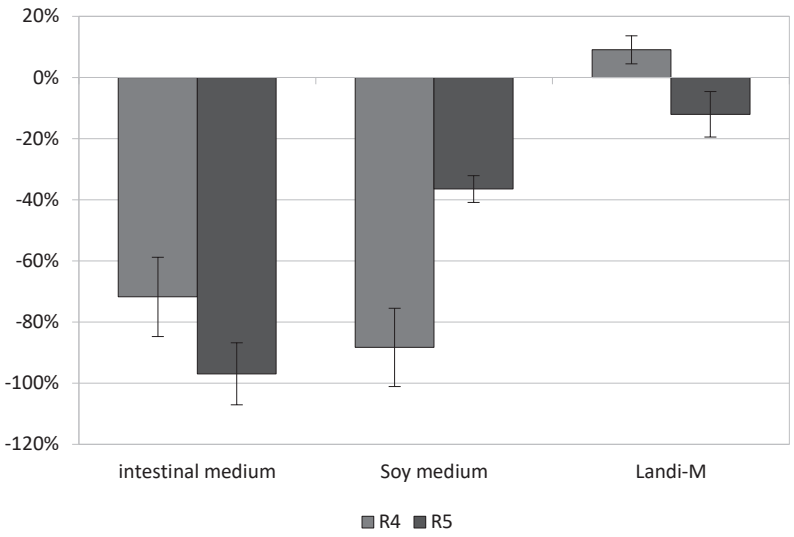

Fig. 3. Changes in bacilisin synthetase gene expression on different media.

Bacilysin synthetase was not detected in strain R1. In strains R4 and R5, a decrease in the level of bacilysin synthetase expression was observed on intestinal and soybean medium. On Landi-M medium, the expression of bacilysin synthetase slightly increased in R4 strain and slightly decreased in R5 strain

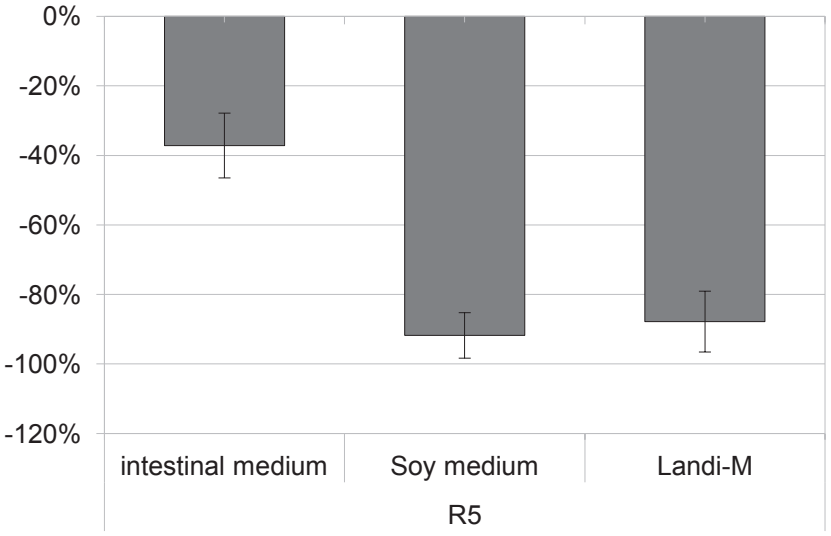

Fig. 4. Changes in fengycin synthetase gene expression on different media**.**

Despite the ability to produce fengycin in all three strains confirmed by mass spectrometry, the fenC fragment of synthetase is expressed only in strain R5. The expression level of fenC on all media is lower than on standard LB.

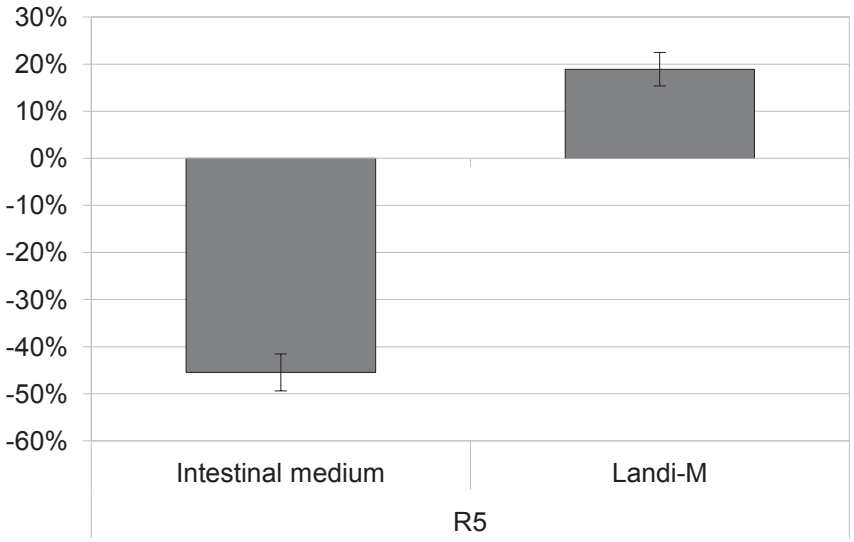

Fig. 5. Variation of iturin synthetase gene expression on different media.

The ituD gene of iturin synthetase is also present only in strain R5. Its expression increases on Landi-M medium, decreases on intestinal medium and is absent on soy medium.

No correlation between the expression level of the negative regulator abrB and the studied NRPS genes was observed, although the expression of abrB decreases on Landi-M medium. Iturin is most efficiently synthesized by strain R5 on Landi-M medium, the expression level of bacitracin synthetase in strain R4, is also higher on Landi-M medium. No expression of surfactin was also observed in the growing conditions on these media.

Thus, it can be seen that the culture medium affects the expression of non-ribosomal synthesis genes. This is consistent with the literature data, in particular, in the study of Geng-Rong Gao [27] an increase in fengycin synthesis by 2.5-fold was observed when threonine amino acid was added to the nutrient medium. Wu Q [28] in their work also achieved a 2.5-fold increase in surfactin titer by adding fatty acids with unbranched chain to the nutrient medium. However, the data on the influence of the medium composition on the level of bacillin expression are not described in the literature.

6 Conclusion

An increase in the expression level of bacillin synthetase on Landi-M medium correlates with a decrease in abrB expression, indicating that abrB is a negative regulator of bacillin synthesis. No correlation was observed between the expression level of abrB negative regulator and the rest of the NRPS genes examined. The expression level of bacillin synthetase is significantly higher on Landi-M medium in all strains. Iturin is most efficiently synthesized by strain R5 on Landi-M medium, the expression level of bacillin

synthetase in strain R4 is also higher on Landi-M medium. Also, it can be said that the expression profile of bioactive metabolites is highly dependent on the medium composition. For several metabolites, modified Lundy medium is the most optimal, but the synthesis of fengycin is higher on LB medium. Thus, in order to obtain target bioactive substances in vitro, it is necessary to take into account the composition of the nutrient medium and modify it to achieve an increase in the synthesis of non-ribosomal peptides.

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