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(54) **Title**: SYNTHETIC GENES ENCODING PEPTIDE FRAGMENTS OF NATURAL MYELIN PROTEINS FOR INDUCTION OF ORAL TOLERANCE, DNA FRAGMENT COMPRISING THESE GENES, MEANS OF OBTAINING THESE PEPTIDES IN A MICROBIAL (BACTERIAL) SYSTEM AND THEIR MEDICAL APPLICATION

(57) **Abstract**: The subject of the invention is the method of producing low molecular weight peptides, derivatives of the spinal cord myelin proteins, in bacteria, in particular lactic acid bacteria, to obtain single and combined preparations of myelin peptides for medical use, in particular for induction of oral tolerance.

Description

Synthetic genes encoding peptide fragments of natural myelin proteins for induction of oral tolerance, DNA fragment comprising these genes, means of obtaining these peptides in a microbial (bacterial) system and their medical application

Technical Field

[0001] The subject of the invention is the method of obtaining synthetic genes encoding low molecular weight myelin proteins, derivatives of protein fragments of mammalian spinal cord, and the method of their microbial production using lactic acid bacteria, single and combined preparations of these peptides and their application in medicine for specific induction of oral tolerance.

Background Art

[0002] Multiple sclerosis is a destructive disease of the central nervous system that affects mainly young people, between 20 and 40 years of age. Incidence data indicate that around 60 000 people suffer from this disease in Poland. It is an autoimmune disease, for which, as yet, there is no effective treatment. Immunomodulatory drugs, such as interferon and Copaxone, only change the course of the disease, reducing the number of attacks, but after 6 years of treatment, the disability of people with multiple sclerosis is the same as without treatment. Moreover, treatment of this disease is quite expensive, and reimbursement of medical expenses – infrequent. Hence the need for intensive research on new effective and economically profitable methods of treating this chronic immune disease.

[0003] Autoimmune diseases are based on a pathological recognition by the immune system of the body's own proteins as foreign antigens, which, in consequence, leads to induction of mechanims eliminating these antigens. In multiple sclerosis, fragments of myelin proteins of the central nervous system constitute antigens pathologically recognized by antibodies.

[0004]

[0005] The mechanism of food tolerance, in which the immune system is specifically desensitized in respect to food nutrients has been made known. It was proposed to use this mechanism for tolerance induction for

protein epitopes in autoimmune diseases, such as rheumatism and multiple sclerosis. The Authors' own studies demonstrate that hydrolysates of the spinal cord of farm animals, containing low molecular weight myelin peptide fragments, exhibit an activity that modulates the course of experimental allergic encephalomyelitis (EAE) in an animal model of multiple sclerosis (MS) (1).

[0006] Undertaking studies aimed at developing a method of producing myelin peptides in lactic acid bacteria was associated with a number of premises: (i) the important status of multiple sclerosis among diseases occurring in Poland; (ii) low efficiency and high costs of medical treatment of this disease; (iii) demonstrated effectiveness of peptides in therapy of this disease unit; (iv) the documented role of the gut (gastrointestinal tract) in the immune system of the organism; (v) previous positive results of using lactic acid bacteria as producers of immunomodulatory factors (cytokines and antigens), including oral vaccines; (vi) data indicating that bacteria intensify the effect of oral tolerance (2).

[0007] CURRENT STATE OF KNOWLEDGE

[0008] The recognized targets in the attack on the immune system in multiple sclerosis and in the experimental allergic encephalomyelitis (EAE) animal model are myelin proteins. Main proteins components of myelin are three proteins:

[0009] (i) myelin basic protein (MBP), which human amino acid sequence is represented by formula **13** (3)

[0010] (ii) proteolipid protein (PLP), which human amino acid sequence is represented by formula **14** (4)

[0011] (iii) myelin-oligodendrocyte glycoprotein (MOG), which human amino acid sequence is represented by formula **15** (5)

[0012] Studies performed during the last decade deliver evidence that peptide fragments, which are products of partial hydrolysis of dietary proteins, have also the ability to penetrate the gut barrier. Some short peptides pentrate this barrier more easily than single amino acids. To differentiate food peptides penetrating the gut-blood barrier from invasive bacterial and viral proteins, organisms have developed a specific system of food antigen

presentation, in result of which the specific immunogenicity for certain peptide sequences (and also other, non-peptide nutrient components) is reduced. This phenomenon has been termed oral tolerance.

[0013] The present invention is based on own preliminary research. Application of a spinal cord hydrolysate preparation in a arbitrarily selected dose in rats with EAE led to reduction of inflammatory symptoms both when applied before and after EAE induction (6).

[0014] Exploitation of lactic acid bacteria as biological factories producing peptide fragments of natural myelin proteins is of great scientific and application interest and presents many beneficial medical and pharmacological aspects.

[0015] Lactic acid bacteria (LAB) constitute a large and taxonomically diverse group of Gram-positive bacteria. An indisputable advantage of these bacteria is their granted GRAS status (Generally Regarded As Safe), i.e. non-pathogenic and safe bacteria for humans and animals. Lactic acid bacteria are a significant object of biotechnological uses as well as basic and applicational research. Biotechnological use of lactic acid bacteria finds application in various industrial branches (7) – food (e.g. production of fermented milk products, food and feeds as well as feed additives), chemical (e.g. production of polymers and enzymes) and pharmaceutical (e.g. probiotic preparation, such as Lakcid or Lactovaginal). In recent years great hopes have been connected with the use of these bacteria in production of functional food, probiotics as well as drugs in the form of oral vaccines (7, 8, 9). In respect to the latter, high expectations are associated with exploiting lactic acid bacteria as vaccine vectors (10-12). Also, over the last few years an increase in research was observed aimed at applying lactic acid bacteria to produce different proteins of therapeutic or prophylactic significance. Lactic acid bacteria are attempted to be used in prophylaxis and treatment of a syndrome known as the inflammatory bowel disease (IBD), which includes also the Crohn's disease and ulcerative colitis.

[0016] The subject of the invention is development of a biological system, based on lactic acid bacteria, for production of peptide fragments of natural

myelin proteins, to create a preparation for induction of oral tolerance.

[0017] Peptide fragments of natural myelin proteins for use in the invention were selected based on own studies, which indicated that patients with multiple sclerosis are identified with the HLA-DR2 antigen that recognizes fragment 84-103 of the MBP protein (13). Antibodies against other fragments of the three main proteins are also identified (14, 15) and during progression of the disease the number of recognized antigens deriving from the three basic myelin proteins increases. The above observations are most probably connected with the availability of these proteins as well as their fragments in patients. Immunogenicity of the selected peptide fragments of natural myelin proteins was confirmed also on EAE animal models (16, 17, 18).

[0018] Unexpectedly, during implementation of the invention it occurred that it is possible to:

1. generate by PCR method synthetic genes encoding selected peptide fragments of natural myelin proteins;
2. clone, in cells of selected strains of Gram-positive lactic acid bacteria from *Lactococcus* genus, the obtained recombinant genes in adequate cloning vectors;
3. express in selected lactic acid bacteria (*Lactococcus, Bifidobacterium, Lactobacillus*) genes encoding peptide fragments of natural myelin proteins;
4. clone genes encoding peptide fragments of natural myelin proteins in cells of Gram-negative bacteria (*Escherichia coli*);

[0019] optimize the biosynthesis of myelin peptides in lactic acid bacteria

Summary of invention

[0020] Synthetic genes encoding peptide fragments of natural myelin proteins for induction of oral tolerance, according to the invention, are characteristic by the fact that they contain at least one nucleotide sequence encoding a myelin protein fragment, selected from a group comprising: nucleotide sequence for MBP21-40 fragment, represented by formula **1**, encoding a peptide of amino acid sequence represented by formula **1a**, and/or nucleotide sequence for MOG35-55 fragment, represented by formula **2**,

encoding a peptide of amino acid sequence represented by formula **2a**, and/or nucleotide sequence for MBP85-97 fragment, represented by formula **3**, encoding a peptide of amino acid sequence represented by formula **3a**, and/or nucleotide sequence for PLP139-151 fragment, represented by formula **4**, encoding a peptide of amino acid sequence represented by formula **4a**, and/or nucleotide sequence for PLP178-191 fragment, represented by formula **5**, encoding a peptide of amino acid sequence represented by formula **5a**.

[0021] The method of generating genes encoding peptide fragments of natural myelin proteins, according to the invention, is based on synthesizing by PCR method of synthetic genes encoding peptide fragments of nautral myelin proteins using 2 complementary primers („LONG") presented in Table 1 specific for each peptide, which nucleotide sequences were designed in such a way so they would correspond to the codons preferably occurring in *Lactococcus lactis* bacteria. Additionally, nucleotide sequences of „FLONG" primers (forward LONG) for peptides MBP85-97, PLP139-151 and PLP178-191 were modified by introducing a sequence corresponding to the translation START codon (ATG), just before the sequence encoding the first amino acid of the original peptide sequence. At the same time the 5' ends of primers „FLONG_peptide" contain an additional, typical for *Lactococcus lactis* bacteria, RBS (ribosome-binding site) sequence recognized by the translation machinery and a 'spacer' sequence (linking sequence) localized between the RBS region and the translation START codon. Primers „LONG" are used as templates for synthesizing by PCR technique the synthetic genes using short primers („SHORT") homologous to the 5' ends of primers „FLONG_peptide" and „RLONG_peptide", where the 5' ends of primers „SHORT" carry additional sequences recognized by specific restriction enzymes, preferably SalI (for forward primer SHORT) and PstI (for reverse primer SHORT), while the 5' ends of prmiers „revSHORT_peptide" (reverse SHORT) have additionally a twice repeated sequence corresponding to the translation STOP codon (TAA) (Table 2).

[0022] The subject of the invention are also DNA fragments obtained through the

above-mentioned PCR reaction using primers „LONG" and „SHORT", which nucleotide sequences, according to the invention, comprise the sequence of synthetic genes of selected peptides of natural myelin proteins (underlined sequences), according to the invention, represented by formula **6**, formula **7**, formula **8**, formula **9** and formula **10.**

[0023] The method of cloning the generated DNA fragments comprising synthetic genes, according to the invention, is based on subjecting them to digestion by restriction enzymes, favorably PstI and SalI, and then ligating them with a plasmid vector that replicates in lactic acid bacteria cells, favorably in *Lactococcus,* in particular with the pIL253 vector, digested beforehand with the same restriction enzymes, to obtain recombinant plasmids, in which the orientation of the cloned PCR products (inserts) is in accordance with the direction of transcription directed from the internal promoter present on the vector, in order to introduce the recombinant DNA by electroporation method into cells of bacterial strains, which are cultured in a known manner. Cells containing the new gene are isolated from the culture population, favorably by analyzing the obtained transformants by PCR technique for the presence of inserts which length corresponds to the length of DNA fragments comprising the expected synthetic genes. Nucleotide sequences of the cloned genes are examined for conformity with the nucleotide sequences of the synthetic genes by DNA sequencig technique.

[0024] In the cloning method according to the invention, it is favorable to use as Gram-positive lactic acid bacterial strains into which recombinant DNA is introduced, *Lactococcus* strains, e.g. *Lactococcus lactis* IL1403, *Lactococcus lactis* IBB477 and *Lactococcus lactis* IBB360, differing in their viability in the mammalian gut as well as other genera of lactic acid bacteria, preferably *Lactobacillus* and *Bifidobacterium.*

[0025] The method of producing selected peptides in a bacterial system, according to the invention, is based on expression of synthetic genes of selected peptides in cells of lactic acid bacteria strains, in particular *Lactococcus,* by culturing them in a known medium specific for lactic acid bacteria, preferably on M17 medium or defined CDM medium (*chemically*

defined medium), supplemented with sugar, favorably glucose or cellobiose, at a concentration no less than 0.5% or on milk, whey or other suitable medium, at a temperature ranging between 20°C-30°C, preferably at 30°C.

[0026] The method of cloning the synthetic genes of selected myelin peptides in Gram-negative bacteria, favorably *Escherichia coli* species, according to the invention, is based on ligation of the synthetic gene amplified using specific primers 'SHORT' (Table 2) with the vector, preferably with a commercially available pGEMT-Easy plasmid or other vector replicating in Gram-negative bacterial cells, favorably from *Escherichia coli* species, to obtain recombinant plasmids. Recombinant DNA is introduced by a known method, e.g. electroporation, to cells of bacterial strains, which are then cultured in a known manner. Cells containing the new gene are isolated from the cultured population, favorably by analyzing the obtained transformants by PCR technique for the presence of inserts which length corresponds to the length of DNA fragments comprising the expected genes. Nucleotide sequences of the cloned DNA fragments are examined for conformity with the nucleotide sequences of the synthetic genes by DNA sequencing technique.

[0027] In the cloning method according to the invention it is favorable to use as Gram-negative bacterial strains, into which recombinant DNA is introduced, bacteria from *Escherichia coli* species, favourably strain TG1.

[0028] The method of optimizing the expression of synthetic genes of selected peptide fragments of natural myelin proteins, according to the invention, favorably is based on replacing the internal, constitutive promoter of the plasmid vector, replicating in *L. lactis* cells, with a regulated promoter, favorably with the promoter region of a gene deriving from the genome of *L. lactis* bacteria or other species or from the genome of a bacteriophage, preferably with the promoter region of *ptcB* gene engaged in sugar catabolism in lactic acid bacteria from *Lactococcus* genus, which is regulated by the presence in the medium of various sugars, such as: cellobiose, galactosa, salicin, esculin, glucose.

[0029] The method of optimizing the production of peptides in a bacterial system,

according to the invention, favorably is based on culturing cells carrying the recombinant plasmid vector (with the cloned synthetic gene and a suitable promoter region, favorably the promoter of *ptcB* gene from the genome of *Lactococcus* bacteria) in a known medium specific for lactic acid bacteria, preferably on defined CDM medium (*chemically defined medium*), supplemented with sugar, favorably glucose or cellobiose, at a concentration of ≥ 0.5%, or on milk, whey or other suitable medium, at a temperature ranging between 20°-30°C, preferably at 30°C.

[0030] The subject of the invention is also the nucleotide sequence of the promoter region of gene *ptcB,* comprising the -10 and -35 region, represented by formula **11**, for optimizing the production of synthetic genes encoding peptide fragments of natural myelin proteins, according to the invention.

[0031] The method of producing the nucleotide sequence of the promoter region of *L. lactisptcB* gene as well as the method of replacing the internal, constitutive promoter of a plasmid vector that replicates in *L. lactis* with the promoter region of *L. lactis ptcB* gene, according to the invention, is based on amplifying by PCR reaction the promoter region of *ptcB* gene using chromosomal DNA of *L. lactis* IL1403 strain as template and pair of primers (ptcBfor and ptcBrev), which sequences are complementary to the DNA sequences flanking the promoter region of the chromosomal *ptcB* gene and modified in such a way that their 5' ends contain sequences recognized by specific restriction endonucleases, preferably VspI (for primer ptcBfor) and NciI (for primer ptcBrev) (Table 3). In result of the PCR reaction, by using such designed primers, according to the invention, a DNA fragment is obtained, which nucleotide sequence is represented by formula **12**, comprising the sequence of the promoter region of *ptcB* gene (underlined).

[0032] The product obtained in this manner is subjected to digestion by restriction enzymes, preferably VspI and NciI, and then ligated with the recombinant vectors carrying synthetic genes encoding the selected peptide fragments of natural myelin proteins, from which the original promoter region was removed (e.g. by digestion using the same restrictases). The recombinant

DNA is introduced by electroporation method into cells of bacterial strains which are then cultured by known means. Cells containing the new promoter region are isolated from the cultured population, favorably by analyzing the obtained transformants by PCR technique for the presence of DNA fragments which expected length corresponds to the length of the selected promoter region of *ptcB* gene. Nucleotide sequences of the cloned DNA fragment are examined for conforminty with the nucleotide sequence of the promotor region of *ptcB* gene, according to the invention, by DNA sequencing technique.

[0033] The subject of the invention is also the application of bacteria producing peptide fragments of natural myelin proteins according to the invention, for induction of oral tolerance.

[0034] Application of the nucleotide sequence of the promoter region of *ptcB* gene, which comprises the -10 and -35 region, represented by formula **11**, according to the invention, for optimizing the production of synthetic genes encoding peptide fragments of natural myelin proteins according to the invention, for induction of oral tolerance.

[0035] It is expected that bacteria producing the peptide fragments of natural myelin proteins, according to the invention, will induce oral tolerance as described in literature referring to oral administration of peptide mix in the form of the animal spinal cord hydrolysate, published in result of own studies (1, 6).

[0036] **Materials and methods**

[0037] Bacterial strains, culture conditions and plasmids used.

[0038] Bacterial strains and plasmids used in the current studies are presented in Table 4. *Lactococcus lactis* strains were cultured in M17 medium (Oxoid, Anglia) supplemented with 0.5 % glucose or in defined CDM medium (*chemically defined medium*) supplemented with glucose or cellobiose (0.5% - 1%) at a temperature between 20°C - 30°C. *Escherichia coli* strains were culture on Luria-Bertani (LB) medium, at a temperature of 37° C. When needed, for selection purposes, the following antibiotic were used: erythromycin 5 μg ml^{-1} for *L. lactis* and ampicillin 100 μg ml^{-1} for *E. coli.*

[0039] Presented below are examples of implementing the invention.

Description of embodiments

[0040] **Example I.**

[0041] Synthetic genes synthesized by PCR method using for each peptide 2 complementary primers (for/rev„LONG") as a template and 2 short primers (for/rev„SHORT") homologous, respectively, to the extremities of the sequences of „LONG" primers (Table 1). In result of the PCR amplification reactions, products of expected length were obtained, which were then digested by enzymes SalI and PstI and ligated with a plasmid replicating in *Lactococcus* bacterial cells – pIL253, digested beforehand with the same enzymes. Subsequently, the two DNA molecules were recombined with each other and introduced by electroporation method into the cells of selected *Lactococcus lactis* strains, which were cultured on solid M17 agar medium supplemented with 0.5% glucose and erythromycin at a final concetration of 5 μg ml^{-1}, and cells containing the expected synthetic genes were isolated from the cultured population by PCR method using specific primers. Nucleotide sequences of the cloned DNA fragments were examined for their conformity with the nucleotide sequences of the synthetic genes by DNA sequencing technique.

[0042] The resulting proper recombinant plasmids were isolated using a known method from *Lactococcus* bacterial cells and introduced by electroporation into cells of other lactic acid bacteria, e.g. *Bifidobacterium* and *Lactobacillus.*

[0043] Cell of *L. lactis* strains were transformed using a known electroporation technique (19). *Bifidobacterium* and *Lactobacillus* cells were transformed using a known electroporation technique, according to previously established procedures (20, 21).

[0044] Other techniques of molecular biology used in the example were in accordance with the standard methodology (22).

[0045] **Table 1**

[0046] Sequence of primers („LONG") used as templates for amplification of sythetic neuropeptide genes.

[0047]

Table 1

Name of neuropeptide	Name of primer	Primer sequence (forward/reverse)
MBP2 1-40	FLONG_MPB21	5' GAGTATTTCTATGGATCATGCTCGTCATGGT TTTTTACCACGTCATCGTGATACTGGTATTT TAGATTCA 3'
	RLONG_MPB21	5' TGAATCTAAAATACCAGTATCACGATGACGT GGTAAAAAACCATGACGAGCATGATCCATA GAAATACTC 3'
MOG3 5-55	FLONG_MOG35	5' GTATTTCTATGGAAGTTGGATGGTATCGTTC ACCATTTTCACGTGTTGTTCATTTATATCGT AATGG 3'
	RLONG_MOG35	5' CCATTACGATATAAATGAACAACACGTGAAA ATGGTGAACGATACCATCCAACTTCCTAGA AATAC 3'
MBP8 5-97	FLONG_MPB85	5' GTATTTCTATGCCAGGATCACGTCCACATTT AATTCGTTTATTTTCACGT 3'
	RLONG_MPB85	5' ACGTGAAAATAAACGAATTAAATGTGGACG TGATCCTGGCATAGAAATAC 3'

PLP1 39-15 1	FLONG _ PLP139	5' GTATTTCTATGCATTCATTAGGAAAATGGTT AGGACATCCAGATAAATTT 3'
	RLONG _ PLP139	5' AAATTTATCTGGATGTCCTAACCATTTTCCT AATGAATGCATAGAAATAC 3'
PLP1 78-19 1	FLONG _ PLP178	5' GTATTTCTATGAATACATGGACAACATGTCA ATCAATTGCTTTTCCATC 3'
	RLONG _ PLP178	5' GATGGAAAAGCAATTGATTGACATGTTGTC CATGTATTCATAGAAATAC 3'

[0048] **Example II.**

[0049] Production of selected peptides in lactic acid bacteria cells was based on expression of synthetic genes in bacterial cells by culturing them on defined CDM medium (*chemically defined medium*), supplemented with cellobiose at a concentration no less than 0.5%, at a temperature ranging between 20°-30°C, preferably at 30°C, until reaching optical density of $OD_{600} \geq 0.6$.

[0050] **Example III.**

[0051] Studies on expression of synthetic genes of selected myelin peptides on the transcriptional level were carried out using the RT-PCR method. Total RNA was isolated from bacterial strains carrying the recombinant plasmids and, after specific treatment, was subjected to reverse transcription reaction using reverse primers (revSHORT_peptide) complementary to the 3' end of the strand encoding the synthetic gene (Table 2). Obatined cDNA was subsequently subjected to amplification by the classical PCR technique using two primer pairs (forSHORTUniv and rev SHORT_peptide) (Table 2). In result of the experiment, two DNA fragments were obtained which length corresponded to the lenght of each of the genes.

[0052] **Table 2**

[0053] Sequence of primers (‚SHORT') used for amplification of synthetic

neuropeptide genes.

[0054]

Table 2

Name of neuropeptide	Name of primer	Primer sequence
Universal forward primer	ForSHORTUniv	5' ACGCGTCGACAAGGAGTATTTCTATG 3'
MBP21-40	RevSHORT_MBP21	5' AACTGCAGTTATTATGAATCTAAAATACCAG 3'
MOG35-55	rev SHORT_MOG35	5' AACTGCAGTTATTATTTTCCATTACGATA 3'
MBP85-97	rev SHORT_MBP85	5' AACTGCAGTTATTAACGTGAAAATAAACG 3'
PLP139-151	rev SHORT_PLP139	5' AACTGCAGTTATTAAAATTTATCTGG 3'
PLP178-191	rev SHORT_PLP178	5'AACTGCAGTTATTATTTTGATGGAAAAGC 3'

[0055] **Example IV.**

[0056] Studies on expression of synthetic genes of selected myelin peptides on the translational level were carried out by immunoblot method, using protein extracts, obtained by known methods from cultures of bacterial cells carrying recombinant plasmids, suspendend in PBS buffer, pH 7.4 and adequate commercially available specific antibodies.

[0057] **Example V.**

[0058] The promoter region of *ptcB* gene was amplified by PCR method using genomic DNA of *L. lactis* IL1403 strain as template and specific primers (Table 3). In result of the PCR amplification, a product was obtained of expected length, which was subsequently digested with NciI and VspI enzymes and ligated with the pIL253 plasmid, replicating in *Lactococcus* bacteria cells, digested beforehand with the same enzymes. The recombinant DNA was introduced by electroporation into cells of *L. lactis* strain, which were cultured on solid M17 agar medium supplemented with 0.5% glucose and erythromycin at a final concentration of 5 μg ml^{-1}, and from the cultivated population cells containing the promoter region of *ptcB* gene were isolated using the PCR method and specific primers.

[0059] Cell of *L. lactis* strains were transformed using electroporation technique (19).

[0060] Other techniques of molecular biology used in the example were in accordance with the standard methodology (22).

[0061] **Table 3**

[0062] Sequence of primers used for amplification of the promoter region of *ptcB* gene.

[0063]

Table 3

Name of primer	Primer sequence
ptcBfor	5' GCGATTAATGTCGCCTAAAGGTTGC 3'
ptcBrev	5' AATCCCGGCGTTTTATAATTTAACGTTC 3'

[0064] **Example VI.**

[0065] Synthetic peptide genes were amplified by PCR method using specific primers 'SHORT' (Table 2) and then ligated with a vector replicating in cells of *Escherichia coli* bacteria - pGEMT-Easy. Recombinant DNA was introdiced by electroporation method into cells of *E. coli* strain, which were cultivated on LB agar medium supplemented with ampicillin at a final

concentration of 100 μg ml^{-1}, and from the cultivated population cells containing the expected synthetic genes were isolated using the PCR method and specific primers. Nucleotide sequences of the cloned DNA fragments were examined for conformity with nucleotide sequences of synthetic genes by DNA sequencing technique.

[0066] *E. coli* cells were transformed using electroporation technique (22).

[0067] Other molecular biology techniques used in the examples were in accordance with the standard methodology (22).

[0068] **Example VII.**

[0069] The structure of the nucleotide sequence of synthetic genes of selected myelin peptides as well as of the promoter region of *ptcB* gene, according to the invention, was confirmed by sequencing of DNA fragments using the BigDye Terminator set (Promega, USA) and ABI377 sequencing apparatus (Applied Biosystem, USA), and recombinant plasmids as template as well as primers complementary to the extremities of each of the inserts. Obtained nucleotide sequences were analyzed using the BLAST program (23).

[0070]

[0071] **Table 4**

[0072] Strains and plasmids.

Table 4

Strain	genotype	source
Lactococcus lactis		
IL1403	Plasmid-free laboratory strain	(24)
IBB360	Strain with potential autolytic properties	J. Bardowski –IBB PAN collection
IBB477	Strain with potential adhesive properties, Tc^r	J. Życka-Krzesińska –IBB PAN collection
Bifidobacterium		
Bifidobacterium	Human intestinal isolate; plasmid-free	IPLA Laboratory Collection

pseudocatenulatum M115		
Lactobacillus		
Lactobacillus plantarum	NCFB1193	NCFB Collection
Escherichia coli		
TG1	supE Q(hsdM-mcrB)(rk-mk-McrB-)thi Q(lac-proAB)F'[traD36 laclq lacZ QM15 proA+B+]	(25)
Plazmid		
pIL253	Ery^r, vector for cloning genes in *Lactococcus* bacteria	(26)
pIL253_p[*ptcB*]	Ery^r, vector for cloning genes in *Lactococcus* bacteria, with regulated promoter region of *ptcB* gene	This work
pGEMT-Easy	Amp^R, vector for cloning genes in *Escherichia* bacteria	Promega

[0073] * Ery^r – erythromycin resistance

[0074] Amp^r – ampicillin resistance

[0075]

Sequence listing free text

[0076] MBP21-40:

[0077] ATGGATCATGCTCGTCATGGTTTTTTACCACGTCATCGTGATACTGGTATTTTAGATTCA

[0078] **formula 1**

[0079] MDHARHGFLPRHRDTGILDS

[0080] **formula 1a**

[0081] MOG35-55:

[0082] ATGGAAGTTGGATGGTATCGTTCACCATTTTCACGTGTTGTTCATTTA TATCGTAATGGAAAA

[0083] **formula 2**

[0084] MEVGWYRSPFSRVVHLYRNGK

[0085] **formula 2a**

[0086] MBP85-97:

[0087] CCAGGATCACGTCCACATTTAATTCGTTTATTTTCACGT

[0088] **formula 3**

[0089] PGSRPHLIRLFSR

[0090] **formula 3a**

[0091] PLP139-151:

[0092] CATTCATTAGGAAAATGGTTAGGACATCCAGATAAATTT

[0093] **formula 4**

[0094] HSLGKWLGHPDKF

[0095] **formula 4a**

[0096] PLP178-191:

[0097] ATGAATACATGGACAACATGTCAATCAATTGCTTTTCCATCAAAA

[0098] **formula 5**

[0099] NTWTTCQSIAFPSK

[0100] **formula 5a**

[0101] **DNA fragment containing synthetic gene of MBP 21-40 peptide**

[0102] 5'ACGCGTCGACAAGGAGTATTTCTATGGATCATGCTCGTCATGGTTTT TTACCACGTCATCGTGATACTGGTATTTTAGATTCATAATAACTGCAGT T3'

[0103] **formula 6**

[0104] **DNA fragment containing synthetic gene of MOG35-55 peptide**

[0105] 5'ACGCGTCGACAAGGAGTATTTCTATGGAAGTTGGATGGTATCGTTC ACCATTTTCACGTGTTGTTCATTTATATCGTAATGGAAAATAATAACTG CAGTT3'

[0106] **formula 7**

[0107] **DNA fragment containing synthetic gene of MBP85-97 peptide**

[0108] 5'ACGCGTCGACAAGGAGTATTTCTATGCCAGGATCACGTCCACATTTAATTCGTTTATTTTCACGTTAATAACTGCAGTT3'

[0109] **formula 8**

[0110] **DNA fragment containing synthetic gene of PLP139-151 peptide**

[0111] 5'ACGCGTCGACAAGGAGTATTTCTATGCATTCATTAGGAAAATGGTTAGGACATCCAGATAAATTTTAATAACTGCAGTT3'

[0112] **formula 9**

[0113] **DNA fragment containing synthetic gene of PLP178-191 peptide**

[0114] 5'ACGCGTCGACAAGGAGTATTTCTATGAATACATGGACAACATGTCAATCAATTGCTTTTCCATCAAAATAATAACTGCAGTT3'

[0115] **formula 10**

[0116] **promoter region of the *ptcB* gene**

[0117] GTCGCCTAAAGGTTGCTATAAAGCGAAAAATAAACTAATTAACAAATTATATAAAGCACTTTCAAAAAATAGATAACGCTTGCATTATGAAAATGAAAACGTTATAATTATTTTTATAAAGAACGTTAAATTATAAAACG

[0118] **formula 11**

[0119] **DNA fragment containing the promoter region of the *ptcB* gene**

[0120] GCGATTAATGTCGCCTAAAGGTTGCTATAAAGCGAAAAATAAACTAATTAACAAATTATATAAAGCACTTTCAAAAAATAGATAACGCTTGCATTATGAAAATGAAAACGTTATAATTATTTTTATAAAGAACGTTAAATTATAAAACGCCGGGATT

[0121] **formula 12**

[0122] **MBP**

[0123] MGNHAGKRELNAEKASTNSETNRGESEKKRNLGELSRTTSEDNEVFGEADANQNNGTSSQDTAVTDSKRTADPKNAWQDAHPADPGSRPHLIRLFSRDAPGREDNTFKDRPSESDELQTIQEDSAATSESLDVMASQKRPSQRHGSKYLATASTMDHARHGFLPRHRDTGILDSIGRFFGGDRGAPKRGSGKDSHHPARTAHYGSLPQKSHGRTQDENPVVHFFKNIVTPRTPPPSQGKGRGLSLSRFSWGAEGQRPGFGYGGRASDYKSAHKGFKGVDAQGTLSKIFKLGGRDSRSGSPMARR

[0124] **formula 13**

[0125] **PLP**

[0126] GLLECCARCLVGAPFASLVATGLCFFGVALFCGCGHEALTGTEKLIETYF

SKNYQDYEYLINVIHAFQYVIYGTASFFFLYGALLLAEGFYTTGAVRQIFG
DYKTTICGKGLSATVTGGQKGRGSRGQHQAHSLERVCHCLGKWLGHP
DKFVGITYALTVVWLLVFACSAVPVYIYFNTWTTCQSIAFPSKTSASIGSL
CADARMYGVLPWNAFPGKVCGSNLLSICKTAEFQMTFHLFIAAFVGAAA
TLVSLLTFMIAATYNFAVLKLMGRGTKF

[0127] **formula 14**

[0128] **MOG**

[0129] MASLSRPSLPSCLCSFLLLLLLQVSSSYAGQFRVIGPRHPIRALVGDEVEL
PCRISPGKNATGMEVGWYRPPFSRVVHLYRNGKDQDGDQAPEYRGRT
ELLKDAIGEGKVTLRIRNVRFSDEGGFTCFFRDHSYQEEAAMELKVEDPF
YWVSPGVLVLLAVLPVLLLQITVGLVFLCLQYRLRGKLRAEIENLHRTFDP
HFLRVPCWKITLFVIVPVLGPLVALIICYNWLHRRLAGQFLEELRNPF

[0130] **formula 15**

[0131]

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[0132]

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[0133]

Claims

1. Synthetic genes encoding peptide fragments of natural myelin proteins for induction of oral tolerance contain at least one nucleotide sequence encoding a fragment of a myelin protein selected from a group comprising: nucleotide sequence for MBP21-40 fragment, represented by formula **1**, encoding a peptide of amino acid sequence represented by formula **1a**, and/or nucleotide sequence for MOG35-55 fragment, represented by formula **2**, encoding a peptide of amino acid sequence represented by formula **2a**, and/or nucleotide sequence for MOG35-55 fragment, represented by formula **3**, encoding a peptide of amino acid sequence represented by formula **3a**, and/or nucleotide sequence for MOG35-55 fragment, represented by formula **4**, encoding a peptide of amino acid sequence represented by formula **4a**, and/or nucleotide sequence for MOG35-55 fragment, represented by formula **5**, encoding a peptide of amino acid sequence represented by formula **5a**.
2. Method of obtaining genes encoding selected peptide fragments of myelin proteins designated for induction of oral tolerance characteristic by the fact that synthetic genes encoding the selected peptides are synthesized by PCR method using 2 complementary starters („LONG") presented in Table 1 specific for each peptide, which nucleotide sequences were designed in such a way so they would correspond to codons preferably occurring in *Lactococcus lactis* bacteria, where the nucleotide sequence of „FLONG" primers (forward LONG) for peptides MBP85-97, PLP139-151 and PLP178-191 was modified by introducing a sequence corresponding to the translation START codon (ATG), just before the sequence encoding the first amino acid of the original peptide sequence, and, at the same time, the 5' ends of primers „FLONG_peptide" contain an additional RBS (ribosome-binding site) sequence typical for *Lactococcus lactis* bacteria, recognized by the translation machinery and a 'spacer' sequence (linking sequence) localized between the RBS region and the translation START codon, where primers „LONG" are used as templates for synthesizing by PCR technique the synthetic genes using short primers („SHORT") homologous to the 5' ends of primers „FLONG_peptide" and „RLONG_peptide", while the 5' ends of primers „SHORT" carry additional sequences recognized by restriction enzymes, preferably SalI (for forward

primer SHORT) and PstI (for reverse primer SHORT), whereas the 5' ends of prmiers „revSHORT_peptide" (reverse SHORT) have additionally a twice repeated sequence corresponding to the translation STOP codon (TAA) (Table 2) to obtain a DNA fragment comprising the synthetic gene sequence represented by formula **1**, formula **2**, formula **3**, formula **4** or formula **5**, encoding a selected peptide fragment of a natural myelin protein.

3. DNA fragments, comprising synthetic gene sequences of selected peptide fragments of natural myelin proteins (underlined sequences) for induction of oral tolerance, which nucleotide sequences are represented by formula **6**, formula **7**, formula **8**, formula **9** and formula **10.**
4. Method of cloning the synthesized DNA fragments comprising synthetic genes, according to claim 1, characteristic by the fact that they are subjected to digestion by restriction enzymes, favorably PstI and SalI, and then ligated with a plasmid vector replicating in lactic acid bacteria cells, favorably *Lactococcus,* in particular with the pIL253 vector, digested beforehand with the same restriction enzymes, to obtain recombinant plasmids, in which the orientation of the cloned PCR products (inserts) is in accordance with the direction of transcription directed from the internal promoter of the vector, in order to introduce the recombinant DNA by electroporation method into cells of bacterial strains, which are cultured in a known manne, and then cells containing the new gene are isolated from the cultured population, favorably by analyzing the obtained transformants by PCR technique for the presence of inserts, which length corresponds to the length of DNA fragments comprising the expected sythetic genes, after which nucleotide sequences of the cloned genes are examined for confromity with nucleotide sequences of the synthetic genes by DNA sequencing technique.
5. Method according to claim 4, characteristic by the fact that preferably used as Gram-positive lactic acid bacterial strains into which recombinant DNA is introduced, are *Lactococcus* strains, e.g. *Lactococcus lactis* IL1403, *Lactococcus lactis* IBB477 and *Lactococcus lactis* IBB360, differing in their viability in the mammalian gut as well as other genera of lactic acid bacteria, preferably *Lactobacillus* and *Bifidobacterium.*
6. Method according to claim 4, characteristic by the fact that synthetic genes of

selected peptide fragments are expressed in cells of lactic acid bacteria strains, in particular in *Lactococcus,* by culturing them in a known medium specific for lactic acid bacteria, preferably in M17 or defined CDM medium (*chemically defined medium*), supplemented with sugar, favorably glucose or cellobiose, at a concentration no less than 0.5%, or in milk, whey or other suitable medium, at a temperature ranging between 20°C-30°C, preferably at 30°C.

7. Method according to claim 4, characteristic by the fact that synthetic genes of selected peptide fragments are expressed in *Escherichia coli* cells, by ligating the synthetic gene, amplified by specific primers 'SHORT' (Table 2), with a vector, preferably with a linearized commercially-available plasmid pGEMT-Easy or other, replicating in Gram-negative bacterial cells, favorably from *Escherichia coli* species, where the recombinant DNA is introduced by a known method, favorably by electroporation, into cells of bacterial strains, which are then cultured by known means, and cells containing the new gene are isolated from the cultured population, favorably through analysis of the obtained transformats by PCR technique for the presence of inserts which length corresponds to the length of DNA fragments comprising the expected synthetic genes, after which nucleotide sequences of cloned genes are examined for conformity with nucleotide sequences of the synthetic genes by DNA sequencing technique.
8. Method according to claim 7, characteristic by the fact that used as Gram-negative bacterial strains, into which recombinant DNA is introduced, are bacteria from *Escherichia coli* species, favourably strain TG1.
9. Method according to claim 4, characteristic by the fact that the promoter region of *ptcB* gene is used to optimize biosynthesis of peptides in lactic acid bacteria.
10. Method according to claim 9, characteristic by the fact that the internal, constitutive promoter of a plasmid vector, replicating in *L. lactis* cells, is replaced by a regulated promoter, favorably by a promoter region of a gene deriving form the genome of *L. lactis* bacteria or other species, or from a bacteriophage genome, preferably by the promoter region of *ptcB* gene engaged in sugar catabolism of lactic acid bacteria from *Lactococcus* genus, regulated by various sugar present in the medium, e.g. cellobiose, galactose,

salicin, esculin, glucose.

11. Method according to claim 9, characteristic by the fact that cells carrying the recombinant plasmid with the cloned synthetic gene and an adequate promoter region, favorably promoter of *ptcB* gene from the genome of *Lactococcus* bacteria, are cultured in a known medium specific for lactic acid bacteria, in particular on defined CDM medium (*chemically defined medium*), supplemented with sugar, favorably glucose or cellobiose, at a concentration of ≥ 0.5%, or in milk, whey or other suitable medium, at a temperature ranging between 20°-30°C, preferably at 30°C.
12. Nucleotide sequence of the promoter region of *ptcB* gene, which comprises -10 and -35 regions, represented by formula **11**, for optimizing the production of synthetic genes encoding peptide fragments of natural myelin proteins according to claim 1.
13. Method of generating the nucleotide sequence of the promoter region of *L. lactisptcB* gene as well as the method of applying it to replace the internal, constitutive promoter of a plasmid vector replicating in *L. lactis* cells, characteristic by the fact that the promoter region of *ptcB* gene is synthetized by PCR reaction using chromosomal DNA of *L. lactis* IL1403 strain as template and pair of primers (ptcBfor and ptcBrev) complementary to the DNA sequences flanking the promoter region of the chromosomal *ptcB* gene, which are modified in such a way that their 5' ends contain sequences recognized by specific restriction endonucleases, preferably VspI (for primer ptcBfor) and NciI (for primer ptcBrev) (Table 3). In result of the PCR reaction, using such primers, a DNA fragment is obtained, which sequence is represented by formula **12**, and which comprises the sequence of the promoter region of *ptcB* gene (underlined), and the resulting product is subjected to digestion by restriction enzymes, preferably VspI and NciI, which is then ligated with the recombinant vectors, carrying synthetic genes endcoding the chosen peptides, from which the original promoter regions have been removed, favorably by using the same restrictases, and finally such recombinant DNA is introduced by electroporation method into cells of bacterial strains, which are cultured by known methods, and cells contaning the new promoter region are isolated from the cultured population, favorably by analyzing the obtained transformants

using the PCR technique for the presence of a DNA fragment of expected length, corresponding to the length of the selected promoter region of *ptcB* gene. Subsequently, the nucleotide sequence of the cloned DNA fragment is examined for conformity with the nucleotide sequence of the promoter region of *ptcB* gene by DNA sequencing technique.

14. Application of bacteria carrying synthetic genes encoding peptide fragments of natural myelin proteins according to claim 1, for induction of oral tolerance.
15. Application of nucleotide sequence of the promoter region of *ptcB* gene, comprising the -10 and -35 region, represented by formula 11, according to claim 12 for optimizing the production of synthetic genes encoding peptide fragments of natural myelin proteins according to claim 1.
16.