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(54) Title: THE METHOD OF BIOTECHNOLOGICAL PREPARATION OF LINCOMYCIN DERIVATIVES AND ITS USING

(57) Abstract: Lincomycin derivatives are produced by biotechnological cultivation of *Streptomyces lincolnensis* ATCC 25466 with at least one gene of the 4-L-propylproline biosynthesis coding cluster inactivated. A substitute of the component whose synthesis is blocked by inactivation is added to the medium. In this way new, biologically active lincomycin derivatives are produced biosynthetically.

The method of biotechnological preparation of lincomycin derivatives and its using

Technical Field

The invention involves uses of mutant strains of lincomycin producers defective in lincomycin biosynthesis for the production of hybrid compounds based on lincosamide antibiotics.

Background of the Art

Due to a permanently increasing number of pathogenic microorganisms resistant to known antimicrobial compounds, the interest in the research and development of new, efficient antibiotics also increases. Combinatorial biosynthesis is one of the ways to produce many potentially efficient compounds. By means of genetic modification of already known genes coding biosynthesis of the active compounds or whole clusters of genes it is possible to obtain a microorganism producing compounds with new structures and new properties. Lincomycin and its derivatives are one of those biologically active compounds. Lincomycin is a metabolite produced by different actinomycete species including the strain of *Streptomyces lincolnensis* used for the treatment of infections caused by grampositive pathogens. Clindamycin is the most common lincomycin derivative and its antimicrobial spectrum has been extended to grampositive anaerobic bacteria and *Plasmodium falciparum* causing malaria. Other lincomycin derivatives were prepared primarily by chemical synthesis. At the same time, enrichment experiments were performed with the strain *Streptomyces lincolnensis* based on adding various precursors to the cultivation medium leading to production of new compounds derived from lincomycin. The new compounds were tested, both *in vitro* and *in vivo,* against different pathogenic microorganisms.

Biosynthesis of lincomycin includes separate synthesis of two precursors, *viz.* 4-L-proline (further PPL) and methylthiolincosamide (MTL) that are condensed to give rise to demethyllincomycin (NDL) which is further methylated to yield the resulting lincomycin.

In 1995 the sequence of the *lmb* cluster of genes coding proteins of the lincomycin biosynthesis in the overproducing strain was described. This cluster contains genes for biosynthesis of both precursors, genes for the condensation reaction, regulatory genes and resistance genes responsible for resistance to the antibiotic. The sequence of the lincomycin cluster of the type strain *Streptomyces lincolnensis* ATCC 25466 has recently been published.

Until now, only the function of proteins LmbB1 and LmbB2 responsible for the first steps of biosynthesis of the PPL moiety of the molecule has been demonstrated experimentally. Functions of the other genes are either unknown or were proposed on the basis of homologies with the BLAST database.

Disclosure of the invention

For the preparation of new compounds based on lincosamide antibiotics it is possible to utilize a collection of strains of lincomycin producers defective in one or more steps of biosynthesis. With this aim in mind mutations in some genes of lincomycin biosynthesis were induced in the type strain *Streptomyces lincolnensis* ATCC 25466. A collection of *S. lincolnensis* strains defective in PPL biosynthesis (genes *lmbA, B1, B2, W, X, Y*) and final methylation (gene *lmbJ*) was prepared.

The strains prepared in this way are to be used for a further testing with respect to the production of new compounds depending also on selected cultivation conditions. According to what is coded by a given gene that has been blocked in the designed strain, substituting components are added, namely derivatives of lincomycin precursors; the biosynthesis is thus not blocked any longer and proceeds giving rise to new, biologically active lincomycin derivatives.

In these strains lincomycin biosynthesis is coded by the *lmb* cluster containing genes *lmbA, B1, B2, W, X, Y* coding for proteins involved in the production of the precursor 4-L-propylproline and gene *lmbJ* coding the final methylation of N-demethyllincomycin. By blocking the 4-L-propylproline production, it is possible to add another proline derivative to the cultivation medium which the microorganism can utilize in the biosynthesis replacing thus 4-L-propylproline. In this way it is possible to prepare a number of new lincomycin derivatives without the use of chemical synthesis.

Figures

Fig. 1: Primers for synthesis of inactivation cassettes

Fig. 2: Chromatogram UPLC analysis, peaks of 4' – butyl- 4'depropyllincomycin (further BuLIN) and 4' – pentyl- 4'- depropyllincomycin (further PeLIN) are detected

Fig. 3: Chemical structure of lincomycin

Fig. 4: The list of the used *E. coli* strains including the contained plasmid and antibiotic present in the cultivation medium and, eventually, the required cultivation temperature.

Fig. 5: The list of the used antibiotics with their abbreviations and concentrations in the cultivation media.

Fig. 6: The list of the used cultivation media with their composition

Examples

Example 1

To obtain a strain *Streptomyces lincolnensis* ATCC 25466 defective in lincomycin biosynthesis one of the genes involved in 4-L-propylproline biosynthesis was inactivated using a kit for directed mutagenesis in streptomycetes (REDIRECT PCR targeting system, Proc Natl Acad Sci USA.100(4). 1541-1546. 2003).

Cosmid LK6 maintained in *E. coli* BW25113/pIJ790, carries the whole *lmb* cluster from *Streptomyces lincolnensis*. In this cosmid the gene *lmbX* was replaced with inactivation cassette *aac(3)IV/oriT* coding apramycin resistance. This inactivation cassette was formed by PCR reaction with the aid of primers Xf and Xr containing homologous flanking regions of gene *lmbX*. A 1383 pb fragment produced by cleaving of plasmid pIJ773 with restriction enzymes EcoR1 and HindIII served as template. *E. coli* BW25113 containing cosmid LK6 and plasmid pIJ790 with genes required for recombination was transformed with the inactivation cassette by electroporation and selected in LB medium for apramycin (50 mg/L), kanamycin (50 mg/L) and carbamycin (100 mg/L) resistance. The cassette was by recombination integrated into cosmid LK6 by exchange for gene *lmbX*.

The cosmid prepared in this way was by conjugation transferred to *S. lincolnensis* ATCC 25466 according to the protocol of Kieser T., Bibb M.J., Chater K.F., Hopwood D.A.: Practical Streptomyces genetics, John Innes Centre, Norwich Research Park, Colney, Norwich, England. (2000)

Transformation of *S. lincolnensis* with cosmid DNA by interspecies conjugation with *E. coli.*

By means of electroporation *E. coli* ET1267/pUZ8002 cells were transformed with cosmid LK6 carrying the inactivation cassette and selected on LB medium with apramycin (50μg/ml) and carbenicillin (100 μg/ml). One of the colonies was inoculated in 10 ml of medium LB containing apramycin (50 μg/ml), chloramphenicol (25 μg/ml) and kanamycin (50μg/ml) and cultivated on a shaker to OD_{600} = 0.4 (about 4 h). The antibiotics were removed from the

culture by washing the cells twice with 10 ml of medium LB (1000 g, 10 min, 20 ^{0} C) and the cells were then resuspended in 1 ml of medium LB.

To 10 µl of spore suspension of *Streptomyces lincolnensis* ATCC 25466 (10^8 cfu) 500 µl of 2 x YT medium were added and the spores were heat-shocked at 50 ^{0} C and then cooled down to laboratory temperature.

0.5 ml suspension of *E. coli* ET12567/pUZ8002 cells was mixed with 0.5 ml of spore suspension of *Streptomyces lincolnensis* ATCC 25466 and centrifuged (10 000 g, 5 s, 20 ^{0}C). The sediment was resuspended in 50 µl of the residual medium. A dilution series from 10^{-1} to 10^{-4} was prepared (suspension was diluted with sterile distilled water). One-hundred µl of each diluted suspension were spread on MS agar with 10 mmol/l $MgCl_2$ without selection and incubated at 30 ^{0}C. After 16 – 20 h each 8 cm Petri dish was evenly overlaid with 1 ml sterile distilled water containing 0.5 mg of nalidixic acid and 1.25 mg of apramycin and further incubated at 30 ^{0} C for 3 – 5 days.

S. lincolnensis clones containing the inactivation cassette in their DNA were selected for apramycin (50 mg/L) resistance, first on MS agar and then on DNA agar.

To eliminate the effect of the inactivation cassette on transcription of genes in the same transcription unit, in LK6 cosmid bearing *E. coli* DH5α the cassette was cleaved off with the aid of FLP recombinase coded by a gene carried on plasmid pBT340. FLP recombinase removed the central part of the inactivation cassette and left an 81 pb DNA region – the so-called "scar" (temperature required for induction recombinase repression is 42 ^{0}C, *E. coli* DH5α cells with plasmid BT340 and cosmid LK6 were therefore cultivated at this temperature).

The mutated scar-bearing cosmid was then transferred by transformation of protoplasts prepared according to the protocol Jandová Z., Tichý P.: Transformation of *Streptomyces lincolnensis* protoplasts with plasmid vectors, in *Folia Microbiologica* (Praha), 37(3), 181 -7. (1992). Resulting cells of the streptomycete strain had functional gene *lmbX* replaced with the inactivation cassette.

Preparation of *S. lincolnensis* protoplasts

Inoculum was prepared by inoculating 50 ml of medium YEME containing 10 % sucrose and 5 mmol/l $MgCl_2$ with *S. lincolnensis* spores bearing the inactivation cassette from an agar slant and incubating on a shaker at 30 ^{0}C for 24 hours. One-ml of the inoculum was then re-inoculated on a fresh YEME medium containing 10 % sucrose and 5 mmol/l $MgCl_2$ 0.5 % glycine and the culture was incubated on a shaker at 28 ^{0}C for 16-17 hours. The cells were

harvested by centrifugation (10 min, 12 ^{0}C, 6000 g) washed twice with 15 ml of buffer P under the same conditions and resuspended in 10 ml of buffer P with lysozyme (2 mg/ml). The cell suspension was incubated at 30 ^{0}C with occasional shaking. Protoplast formation was followed under the microscope. Mycelium residues were removed by centrifugation (10 min, 20 ^{0}C, 1 000 g). Protoplasts were filtered through a sterile cotton filter, centrifuged (10 min, 20 ^{0}C, 3 000 g) and washed twice with buffer P under the same conditions. The sedimented protoplasts were finally resuspended in 200 µl of buffer P and immediately used for transformation.

Transformation of *S. lincolnensis* protoplasts

Five-hundred ng in a volume of 5 µl plasmid DNA of the mutated scar bearing plasmid were mixed with 5 µl of 50 % sucrose and 100 µl of the protoplast suspension were added to the mixture. After mixing 200 µl of buffer T were added, the protoplast suspension was mixed again and immediately spread on R2YE agar. After a 16-h incubation at 30 ^{0}C both Petri dishes were overlaid with 2 ml of aqueous kanamycin (200 µg/ml of R2YE agar) and incubated at 30 ^{0}C for additional 7-10 days.

The inactivation cassette was exchanged by the scar by homologous recombination. Double recombinants of the obtained *Streptomyces lincolnensis ΔlmbX* strain were selected for the loss of apramycin resistance.

E. coli cells were cultivated in liquid or solid media (LB, SOC) at 37 ^{0}C. The strain BW25113/LK6/pIJ790 was cultivated at 30 ^{0}C. After transformation with the cassette the strain was cultivated at 37 ^{0}C. The strain DH5α containing plasmid pBT340 was cultivated at 30 ^{0}C. Liquid cultures were incubated on a rotary shaker at 200 rpm.

Streptomyces lincolnensis cells were cultivated in liquid or solid media (R2YE, MS, DNA, YT) at 28 – 30 ^{0}C. Liquid cultures were incubated on a rotary shaker at 230 rpm and stored in 500-ml Erlenmeyer flasks with inserts preventing formation of mycelium clumps. After selection of cells carrying the vector appropriate antibiotics were added to the cultivation media.

Example 2

Streptomyces lincolnensis ΔlmbX defective in PPL biosynthesis was at a concentration of 10^8 spores inoculated in 50 ml of inoculation medium YEME and cultivated at 28 ^{0}C for 30 hours. Twenty-ml of production medium AVM containing 4-L-butylproline (100 mg/l) were then inoculated with 5 % of the inoculation culture. The culture was then cultivated for 120 hours at 28 ^{0}C . The culture liquid was separated from the grown culture by centrifugation and the supernatant was used for the analysis of the produced lincomycin derivative – BuLIN.

Example 3

Streptomyces lincolnensis ΔlmbX defective in PPL biosynthesis was at a concentration of 10^8 spores inoculated in 50 ml of inoculation medium YEME and cultivated at 28 ^{0}C for 30 hours. Twenty ml of production medium AVM containing 4-L-pentylproline (100 mg/l) were then inoculated with 5 % of the inoculation culture. The culture was then cultivated for 120 hours at 28 ^{0}C . The culture liquid was separated from the grown culture by centrifugation and the supernatant was used for the analysis of the produced lincomycin derivative – PeLIN.

Example 4

Acquity UPLC system (Waters, Milford, Massachusetts) with 2996 PDA detector set at 194 nm was used for the analysis of BuLIN. The results were evaluated by computer program Empower 2 (Waters). Supernatant of cultivation medium was purified on SPE Oasis HLB 3cc extraction columns. The sample (4 ml) was applied to the column preconditioned and equilibrated with methanol (3 ml) and water (3 ml). A part of the sample matrix was washed with water (3 ml) and 10 % methanol (v/v, 1 ml). Fractions containing lincomycin derivatives were eluted with 80 % methanol (v/v, 1 ml), evaporated to dryness and re-diluted in 200 µl methanol. Samples were analyzed on a chromatography column BEH C18 (50 x 2.1 mm, particle diameter 1.7 µm, Waters); mobile phase consisted of components A, 1 mmol/l amonium formiate (pH 9.0) and B, acetonitrile; isocratic elution (24 % B); flow rate: 0.4 ml min^{-1}, column temperature: 35 ^{0}C , data collection rate: 20 pts s^{-1} , dose volume: 5 µl, analysis time: 3.5 min. After each analysis the column was washed (100 % acetonitrile, 1 min) and equilibrated (1.5 min). Eluate containing BuLIN (retention time 5.05 min) was collected, evaporated to dryness and used for MS analysis which confirmed the presence of BuLIN in the sample.

Example 5 .

Acquity UPLC system (Waters, Milford, Massachusetts) with 2996 PDA detector set at 194 nm was used for the analysis of PeLIN. The results were evaluated by computer program Empower 2 (Waters). Supernatant of cultivation medium was purified on SPE Oasis HLB 3cc extraction columns. The sample (4 ml) was applied to the column preconditioned and equilibrated with methanol (3 ml) and water (3 ml). A part of the sample matrix was washed with water (3 ml) and 10 % methanol (v/v, 1 ml). Fractions containing lincomycin derivatives were eluted with 80 % methanol (v/v, 1 ml), evaporated to dryness and re-diluted in 200 µl methanol. Samples were analyzed on a chromatography column BEH C18 (50 x 2.1 mm, particle diameter 1.7 µm,

Waters); mobile phase consisted of components A, 1 mmol/l amonium formiate (pH 9.0) and B, acetonitrile; isocratic elution (24 % B); flow rate: 0.4 ml min^{-1}, column temperature: 35 ^0C , data collection rate: 20 pts s^{-1} , dose volume: 5 µl, analysis time: 3.5 min. After each analysis the column was washed (100 % acetonitrile, 1 min) and equilibrated (1.5 min). The eluate containing PeLIN (retention time 5.05 min) was collected, evaporated to dryness and used for MS analysis which confirmed the presence of PeLIN in the sample.

Example 6

With the aim of obtaining *Streptomyces lincolnensis* ATCC 25466 defective in propylproline biosynthesis and final N-demethyllincomycin methylation, genes *lmbX* and *lmbJ* were simultaneously inactivated with the aid of the kit for directed mutagenesis of streptomycetes (REDIRECT OCR targeting system, Proc Natl Acad Sci U S A, 100(4), 1541-1546, 2003). In cosmid LK6 carrying the whole *lmb* cluster gene *lmbX* was replaced with apramycin resistance (50 mg/L) coding inactivation cassette *aac(3)IV/oriT*. The inactivation cassette was prepared by means of PCR reaction with primer Xf and Xr (table 2) containing homologous flanking regions of gene *lmbX*. A 1383 pb fragment formed by cleaving of plasmid pIJ773 with restriction enzymes EcoRI and HindIII served as template. In the same cosmid additional gene *lmbJ* was replaced with a *vph* cassette coding viomycin (30 mg/L) resistance. The inactivation cassette was prepared by PCR reaction using primers Jf and Jr containing homologous flanking regions of gene *lmbJ*. A 1622 pb fragment formed by cleaving of plasmid pIJ781 with restriction enzymes EcoRI and HindIII served as template. *E. coli* BW25113 containing cosmid LK6 and plasmid pIJ790 with genes required for recombination was gradually transformed with inactivation cassettes. By recombination the cassettes were integrated in cosmid LK6 in exchange for genes *lmbX* and *lmbJ*. The cosmid prepared in this way was by conjugation transferred to *S. lincolnensis* ATCC 25466. The cassettes containing *S. lincolnensis* clones were selected for apramycin and viomycin resistance. To eliminate the effect of the inactivation cassettes on transcription of genes in the same transcription unit the cassettes were cleaved off by FLP recombinase coded by a gene carried on plasmid pBT340. FLP recombinase removed central parts of the inactivation cassettes and left 81 pb long DNA regions – "scars". The mutated cosmid with scars was then introduced to a streptomycete strain in which genes *lmbX* and *lmbJ* had already been replaced with the inactivation cassettes. By homologous recombination the inactivation cassettes were exchanged for the scars. Cultivation, selection and transformation conditions were identical with those described in Example 1.

Industrial Applicability

The newly prepared derivatives of lincomycin antibiotics can be used for the development of new biologically active compounds against infections caused by pathogenic microorganisms, *i.e.* in pharmaceutical industry and medicine.

C L A I M S

1. The method of biotechnological production of lincomycin derivatives characterized in that the strain *Streptomyces lincolnensis* ATCC 25466, in which at least one gene of the *lmb* cluster of genes coding for 4-L-propylproline has been inactivated, whereas a substituent of a component whose synthesis is blocked by inactivation must be added to the cultivation medium.
2. The method of biotechnological production of lincomycin derivatives according to claim 1 characterized in that gene *lmbX* coding for biosynthesis of 4-L-propylproline is inactivated.
3. The method of production of lincomycin derivatives according to claim 2 characterized in that a proline derivative is added to the medium as a substitute for a component.
4. The using of the strain according to claim 1 for biotechnological production of biologically more active lincomycin derivatives.
5. The using of the strain according to claim 4 with inactivated genes *lmbJ* and *lmbX* for biotechnological production of biologically more active lincomycin derivatives.
6. The using of the strain according to claim 4 with inactivated gene *lmbX* for biotechnological production of biologically more active lincomycin derivatives.
7. The using of the strain according to claim 4 with inactivated gene *lmbJ* for biotechnological production of biologically more active lincomycin derivatives.

Fig.1

Inactivation primers

dJf
5‘-cgagtggccaacgtcctggtgaggaaagagaattcaatgattccggggatccgtcgacc- 3‘

dJr
5‘-gcgccctagtcaccgtgcgcccgggcgtagacggacacgtgtaggctggagctgcttc- 3‘

dXf
5‘-cgcgcccatcctgcacagcgcaccggaggaagcatgatcattccggggatccgtcgacc- 3‘

dXr
5‘-gagaaaagagccgctgacgcaaggggccctcggcgactatgtaggctggagctgcttc- 3‘

Verification primers

chJf 5‘-tcccggtcgaagaacac- 3‘
chJr 5‘-gtcgcgccctagtcac- 3‘
chXf 5‘-ccggcatcaacgact- 3‘
chXr 5‘-ccagatggaacgaattca- 3‘

Fig. 2

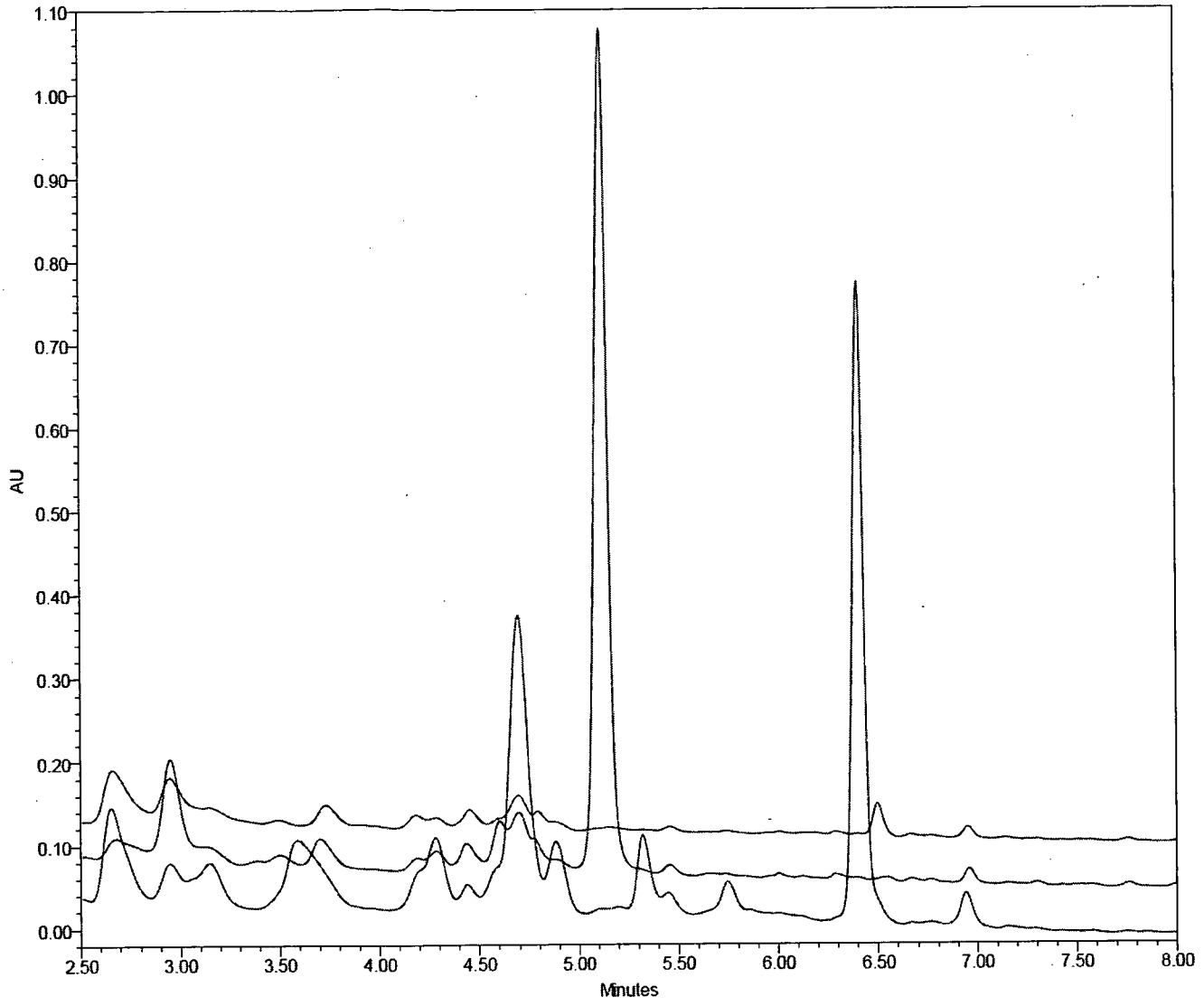
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
AU
2.50
3.00
3.50
4.00
4.50
5.00
5.50
6.00
6.50
7.00
7.50
8.00
Minutes

Fig. 3

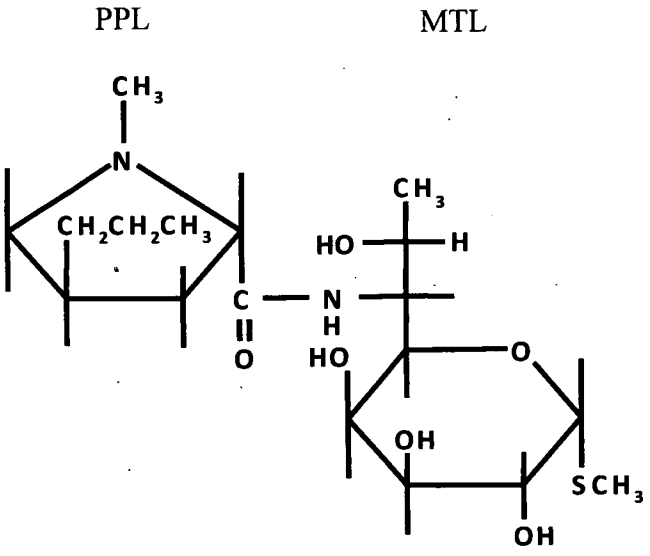

Fig. 4

E. coli strains used:

Bacterial strain/plasmid/antibiotic:

- DH5α/pIJ773/apra and amp
- DH5α/pIJ781/viomycin
- BW25113/pIJ790/cm **(30 °C)**
- BW25113/pIJ790+LK6 cosmid/karb + cm + kan **(30 °C)**
- BW25113/ LK6 cosmid+cassette pIJ773/ apra + karb + kan
- BW25113/ pIJ790+LK6 cosmid+cassette pIJ773/ apra + cm + kan **(30 °C)**
- BW25113/ LK6 cosmid+cassette pIJ773+cassette pIJ781/ apra + karb + kan + vio
- ET12567/pUZ8002/kan (25 μg/ml) + cm
- ET12567/pUZ8002+ LK6 cosmid+cassette pIJ773/apra + cm + kan
- DH5α/BT340/cm **(30 °C)**
- DH5α/LK6 cosmid with scar/kan

Fig. 5

Selection antibiotics:

Antibiotic concentration in the medium:

- Apramycin (apra) 50 μg/ml
- Ampicillin (amp) 100 μg/ml
- Carbenicillin (karb) 100 μg/ml
- Viomycin (vio) 30 μg/ml
- Chloramphenicol (cm) 25 μg/ml
- Kanamycin (kan) 50 μg/ml

Fig. 6

LB medium	
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
dH_2O	ad 1 000 ml, pH 7,5

SOC medium:	
Yeast extract	5,5 g
Tryptone	20 g
NaCl 1 M	10 ml
KCL 1 M	2,5 ml
dH_2O	970 ml, pH 7,0
Added after sterilization	
2 M $MgCl_2$	10 ml
2 M glucose	10 ml

YEME :	
Yeast extract	3 g
Peptone	5 g
Malt extract	3 g
Sucrose	340 g
Glucose	10 g
dH_2O	up to 1 000 ml, pH 7,2
Added after sterilization:	
2,5 M $MgCl_2$	2 ml

DNA agar:	
Agar	10g
Tryptone	10g
NaCl	5g
Glucose	1g
dH_2O	up to 1 000 ml

2 x YT medium:	
Difco Bacto tryptone	16 g
Difco Bacto yeast extract	10 g
NaCl	5 g
dH_2O	up to 1 000 ml

LB agar	
LB medium	1 liter
Agar	15 g

MS agar:	
Agar	20 g
Mannitol	20 g
Soya meal	20 g
Tap H_2O	up to 1 000 ml

BG agar:	
Yeast extract	4 g
Malt extract	10 g
Glucose	4 g
$CaCO_3$	2 g
Agar	12 g
dH_2O	up to 1 000 ml, pH 7,2

B1 agar:	
Beef extract	10 g
Peptone	10 g
NaCl	5 g
Agar	20 g
dH_2O	up to 1000 ml, pH 7,2

R2YE agar:	
Sucrose	103 g
K_2SO_4	0,25 g
$MgCl_2$ $6H_2O$	10,12 g
Glucose	10 g
Casamino acids	0,1 g
Agar	20 g
dH_2O	up to 800 ml, pH 7,2
Added after sterilization:	
Yeast extract (10%)	50 ml
KH_2PO_4 (0,5%)	10 ml
$CaCl_2$ $2H_2O$ (3,68 %)	80 ml
L-proline (20%)	15 ml
TES (5,73%, pH 7,2)	100 ml
Trace elements	2 ml
1M NaOH	5 ml

T buffer:	
Sucrose	25 ml
H_2O	75 ml
Trace elements (chapt. 3.1.3.)	0,2 ml
K_2HPO_4 (2,5%)	1 ml
To 9,3 ml mixture added:	
$CaCl_2$	0,2 ml
1M Tris/maleinic acid, pH 8,0	0,5 ml

Trace elements :	
$ZnCl_2$	40 mg
$FeCl_3$. 6 H_2O	200 mg
$CuCl_2$. 2 H_2O	10 mg
$MnCl_2$. 4 H_2O	10 mg
$Na_2B_4O_7$. 10 H_2O	10 mg
$(NH_4)_6Mo_7O_{24}$. 4 H_2O	10 mg
dH_2O	up to 1 000 ml

P buffer :	
Sucrose	103 g
K_2SO_4	0,25 g
$MgCl_2$. $6H_2O$	2,02g
Trace elements (chapt. 3.1.3.)	2 ml
Filled with H_2O up to 800ml	
After sterilization added to 50 ml:	
K_2HPO_4 (0,5%)	1 ml
$CaCl_2$. $2H_2O$ (3,68%)	10 ml
TES buffer (5,73%, pH 7,2)	10 ml

AVM medium:	
$(NH_4)_2 SO_4$	2 g
Yeast extract	2 g
NaCl	2g
K_2HPO_4	50 mg
$CaCO_3$	5 g
$FeSO_4.7H_2O$	50 mg
$MnSO_4.7H_2O$	50 mg
$MgSO_4.7H_2O$	100 mg
dH_2O	up to 880 ml, pH 7,4
Added after sterilization::	
25% glucose	120 ml