International Journal of Advanced Biotechnology and Research (IJBR)
ISSN 0976-2612, Online ISSN 2278–599X,
Vol-7, Issue-2, 2016, pp755-759
http://www.bipublication.com

Research Article

Cloning and expression of hygromycin resistance gene (hph) from *Streptomyces* to *Lactobacillus acidophilus*: A new clone candidate as an additive to livestock food.

Rebah N. Algafari

[Received-09/06/2016, Accepted-22/06/2016, Published-30/06/2016]

ABSTRACT

About 65 local *Streptomyces* isolates were tested for the presence of hygromycin gene using a rapid PCR dependent method utilizing specific primers designed for this purpose. Only three isolates were found to harbor such gene, and one of them that showed high biological activity was selected for resistance gene (*hph*) isolation and sequencing. The sequence was similar to that on the expression vector pTKIP-*hph* which was used to transform *Lactobacillus acidophilus* with (*hph*). The transformation efficiency was 0.9 x 106, and this bacterium was able to grow on media with 100µg/ml hygromycin as a selective agent. Real-Time PCR amplification showed that (*hph*) gene was expressed with high efficiency in the transform ants.

Key words:*Streptomyces*, hygromycin, *Lactobacillus* transformation, expression vector, Real-Time PCR.

1.INTRODUCTION

Hygromycin B is an aminocyclitol antibiotic produced by *Streptomyces* hygroscopicus which inhibits protein synthesis in both prokaryotes and eukaryotes by interfering with ribosomal translocation and with aminoacyl-tRNA recognition (Blochlinger and Diggelmann 1984).

hygromycin B is produced by *Streptomyces* hygroscopicus and specifically blocks the translocation step on both 70s and 80s ribosomes. The drug also induces misreading both in vivo and in vitro and therefore promotes phenotypic suppression. S. hygroscopicus contains a phosphotransferase (*hph*) activity which phosphorylates hygromycin B, thus potentially providing the producing organism with autoimmunity against the toxic effects of the drug (Pardo et al., 1985, Borovinskayaet al., 2008).

Aminocyclitols, which are characterized by the presence of a cyclohexane moiety with hydroxyl and amino or guanidine substituents, are found in a large class of natural products withbroad-ranging biological properties. The aminocyclitol-aminoglycosideshave long been known for their antibacterial activitiesand have found applications as antibiotics in clinical use (Palaniappanet al., 2009). However, *hph* gene was cloned in several organisms like *E. coli* (Zalacainet al., 1987), yeast (Vickers et al., 2013), and as a selective marker in plants (Soltanmohammadiet al., 2014). The concept of using*Lactobacillus* species as probiotic for disease treatment and prevention as well as health restoration and maintenance is not new. There has been a renewal of interest in the use of probiotics (as distinct from antibiotics) (also termed bio therapeutic agents), driven in large part by consumers and the lay press. Probiotics have been used therapeutically to modulate immunity, lower cholesterol, treat rheumatoid arthritis, prevent cancer, improve lactose intolerance, and prevent or reduce the effects of atopic dermatitis, Crohn's disease, diarrhea, and constipation as well as candidiasis and urinary tract infections (UTI)

(Reid, 1999). The main idea of this research is to produce a clone with both properties; hygromycin resistance and as producer for probiotics; that make it a candidate as an additive to live stock feed.

2. MATERIALS AND METHODS

2.1. Bacterial isolates

All bacterial isolates are grown and preserved at Biotechnology Research Center, Al-Nahrain University. *Lactobacillus acidophilus* was identified by bioMérieux's API®, while *Streptomyces* isolates were previously isolated and identified by Al-Gafari 2014. The expression vector pTKIP-*hph* was harbored by *E. coli*DH5alpha that was provided byAddgen Company.

2.2.Isolation of genomic DNA, RNA, Expression vector

DNA from *Streptomyces* was isolated using Favorgen FATGK 001-1 Tissue genomic DNA extraction mini kit, while RNA from *Lactobacillus acidophilus* and *E. coli* DH5alpha was isolated using Favorgen FABRK 001-1 total RNA mini kit. Expression vector was extracted using Favorgen FABDE 300 plasmid extraction kit from *E. coli*.

2.3. Primers and PCR amplification parameters

Conventional PCR primers and conditions

The following primers were designed in this study using primer blast software http://www.ncbi.nlm.nih.gov/tools/primer-blast/and was manufactured and provided by www.synthesisgene.com.

Primer name	Primer sequence	fragment length		Position
HYGV-1-F	AGCGCGATGTTCTCCATGAT	456		7552
HYGV-1-R	CTATCTCCGGCGCTTCTACG			
HYGV-2-F	CGTAGAAGCGCCGGAGATAG	640		7997
HYGV-2-R	TACGCGTTCTTCCGGATCTC			
HYGD-1-F	TCGTATCCGCCAATGAGTCG	454		13772
HYGD-1-R	TTGCGTTCGGAGACGAAGAA			
HYGD-2-F	TGCTGTTCGTATCCGCCAAT	309		13766
HYGD-2-R	CGAGTCCCTCGTAGTTGTCG			
HYGN-1-F	TTCAGCGACCAGTGCTCATT	327		22888
HYGN-1-R	GGGCAAGCAGGACCACTATC			
HYGN-2-F	AGCGACCAGTGCTCATTGAC	323		22891
HYGN-2-R	GGCAAGCAGGACCACTATCT			
HYGN-3-F	AGATAGTGGTCCTGCTTGCC	385		23194
HYGN-3-R	ACCTGCCGAGCTACTCCTC			
HYGN-4-F	CTTCAGCGACCAGTGCTCATT	318		22887
HYGN-4-R	GACCACTATCTGGCGGCGTA			
HYGN-5-F	TGACCAGATGTCCGATCGCC	306		22907
HYGN-5-R	GCAAGCAGGACCACTATCTGG			
HYGZ-1-F	GCTTCCTCGTATGCGTCGTA			30553
HYGZ-1-R	CAGATCTGGATCCGCCTCAC			
HYGZ-2-F	GTGGATCCAGGGGATTTCGG			30933
HYGZ-2-R	CGAGAAAGACGCCTGCTACA			

PCR amplification conditions were: initial denaturation at 94^0C for 5 min, followed by 35 cycles with 95^0C for 30 sec, 55^0C for 30 sec, 72^0C for 45 sec. after that final extension at 72^0C for 7 min.

2.4.Real-Time PCR primers and conditions

The following primers and probe was designed during this study using rescript Real-Time PCR primer design tool https://www.genscript.com/ssl-bin/app/primer and was manufactured by Synthesis gene Company.

Name	Sequence	Strand	Position	Tm °C	Modification
HygnRT-1	ACCAACATCTTCGTGGACCT	forward	756	59.43	
HygnRT-2	CATAGACGTCGGTGAAGTCG	reverse	803	59.31	

HygnRT-P	CGATCCCGGTGACCTCGGTC	reverse	782	69.50	5'Fam - 3'Tamra

Amplicon Size = 67

The amplification was carried out after reverse transcription of RNA from both *L. acidophilus* and *E. coli* DH5alpha using synthesis gene complete reverse transcription kit ZP00602(5000U) following this protocol:

48℃30 min→80℃2 min→4℃ hold, afterward Real-Time amplification were as follow using Applied Bio system 7200 thermo cycler: 50°C 2 min in 1 cycle, 95°C 10 min in 1 cycle, 95 °C 15 s → 60 °C 30 s → 72 °C 30 s in 40 cycles, finally 72°C 10 min in 1 cycle.

2.5.Electroporation and transformation of pTKIP-*hph* into *L. acidophilus*

Electroporation was done in *L. acidophilus* according to Protocol-No.-4308-91 by Eppendorf using Eppendorf Operator.

3. RESULTS

Hygromycin is an antibiotic that was discovered in 1950s and been a compound of interest since that time for its important application in agriculture, veterinary, as well as a selective agent in immunity research and cell culture. The resistance gene *hph* was isolated and cloned for detailed study, and the major recipient for this gene was *E. coli*, and many cloning vectors were made for this purpose. However, our idea for the combination of probiotic and hygromycin as additives in livestock feed was behind this research to make a clone with both properties. The expression vector pTKIP-*hph* has ORFs that can be recognized by *Lactobacillus* which made it a candidate to be used as an expression vector in this bacterium.

3.1.Detection of resistance gene *hph* in *Streptomyces*

A set of primers designated as HygV, HygD,HygZ, andHygN were designed depending on gene sequence of hygromycin. The HygN primers were designed to recognize the sequence of *hph* in *Streptomyces*, while the other sets amplified different positions of hygromycin gene. Figure 1 shows results of PCR amplification for hygromycin gene from DNA obtained from *Streptomyces*.

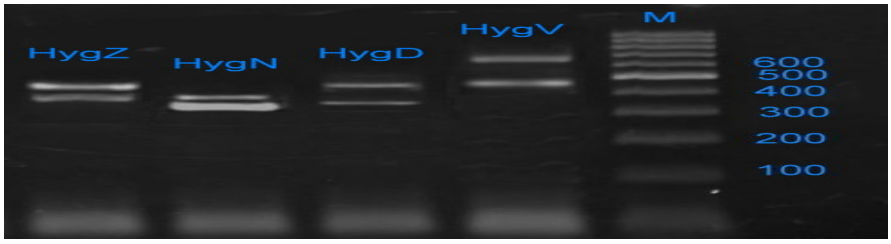

Figure (1). Amplification of hygromycin gene from *Streptomyces* using primers designed in this study. Electrophoresis was carried out at 2% agarose for 90 min with 10v/cm field strength.

The use of specific PCR dependent method for the detection of hygromycin gene is very reliable and we were able to find the isolates carrying the gene among 65 one in a short time with less effort and high precision by observing fragments generated after the amplification is complete.

4.2.Transformation in *L. acidophilus* and selection of trans formants: Electroporation based transformation method prove to be one of the most successful methods used for this purpose. The main parameters that should be considered to obtain high transformation ratio is that should be done in highly aseptic conditions, high concentration of transformation materials in relation to transformed organism, and the trans formants should be immediately cultivated in warm and complete medium eliminate the stress made upon them due to electric potential and restore their cell membrane. The selection of transformed bacteria depended on positive selection method. *Lactobacillus* used in this study was highly sensitive to hygromycin. A minimum

concentration of 10µg was able to inhibit colony growth completely, while transformed bacteria were able to grow in a medium with 100µg of hygromycin which may indicate that *hph* gene in them is highly and efficiently expressed. Confirmation of transformation was made in two steps. The first one is to isolate the expression vector from transformed bacteria and compare that with the original one isolated from *E. coli*DH5alpha as shown in figure 2.

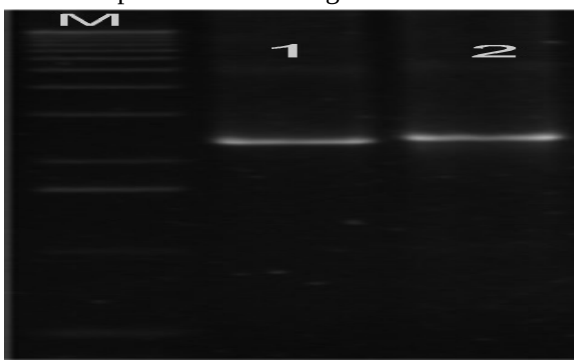

Figure 2. Expression vector isolated from (1) *E. coli* DH5alpha, (2) from *L. acidophilus*. The marker used is 1Kbp-10Kbp.

Further confirmation was made by amplifying *hph* gene from expression vector isolated from *L. acidophilus* using HygN-2, HygN-3, and HygN-5 as shown in figure 3.

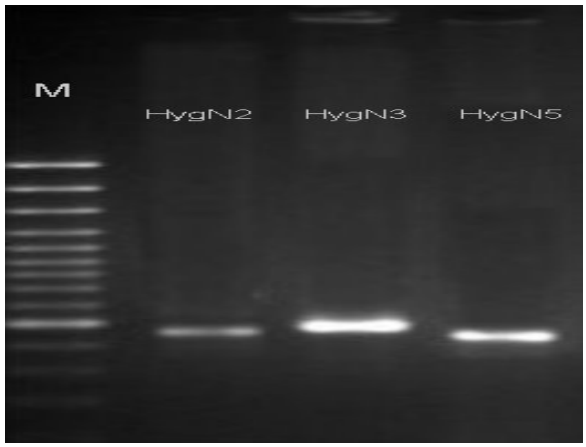

Figure 3. PCR amplification of expression vector pTKIP-*hph* isolated from transformed *L. acidophilus* using primers HygN-2,HygN-3, and HygN-5.

4.3.Expression of pTKIP-*hph* in *L. acidophilus*

Transformation in *L. acidophilus* was successful and selection of transformed cells was done using positive selection by growing those cells on media with hygromycin which was originally sensitive to it at low concentration. Level and efficiency of expression was made by comparing expression of the vector in *L. acidophilus* to *E. coli* DH5alpha. Figure 4 shows data obtained from Real – Time amplification of *hph* in both bacteria. The figure shows that there is a similarity in the level of expression of *hph* which may explain the new trait of hygromycin resistance acquired by transformed *L. acidophilus*.

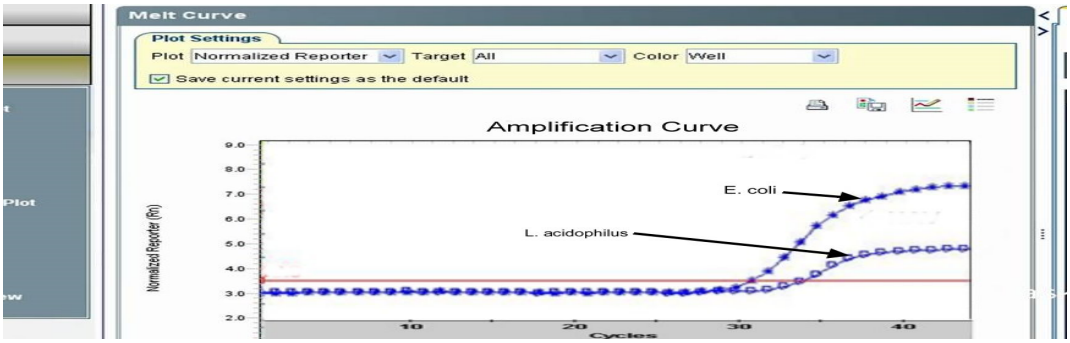

Figure 4.Real time amplification of cDNA from *E. coli*DH5alpha and *L. acidophilus*. The figure shows proximity in expression of *hph* gene in both bacteria.

5.DISCUSSION

Hygromycin since its discovery in 1950s till this day being used for pre immunization of animals against viral, bacterial, fungal, and viral infection. This is because it is a wide spectrum antibiotic and has the ability to inhibit protein synthesis which prevents pathogens from colonization within the host, and eventually causing the disease. *Streptomyces* is the main producer of this compound and it was successfully isolated from

Streptomyceshygroscopicus. Production of such substance with inhibitory trait requires the organism to resist its lethal effect. This was achieved with the presence of phosphorylating enzyme coded by specific gene within the structure of hygromycin operon. This gene (*hph*) was isolated and sequenced and given the name N gene had been cloned in many occasions in *E. coli* for detailed study. However, no literature was found stating that it was cloned in lactobacilli. The use of PCR based method for the identification of *Streptomyces* that harboring hygromycin gene was very efficient, low cost, and less time consuming with high accuracy especially when there is 65 isolates of *Streptomyces*need to be investigated for the presence of hygromycin gene.The use of pTKIP-*hph* was successful choice to transfer *hph* gene due to the similarity in ORFs and the ability of *L. acidophilus* to recognize the promoter sequence of *hph* gene and presence of the mechanism to translate its RNA to the phosphotransferase enzyme which gave them the immunity against hygromycin lethal effect

6.ACKNOWLEDGMENT

Faithful thanks to Addgen company members for the great help and support they provided during preparation for this research.

REFERENCES

1. Al-Gafari, R.N. 2014. Assessment of profile depth, site of sampling, type of media and methods used for the isolation of actinomycetes. International Journal of Microbiology Research. 6, p:553-558.
2. Blochlinger; K. and Diggelmann;H, 1984. Hygromycin B Phosphotransferase as a Selectable Marker for DNA Transfer Experiments with Higher Eucaryotic Cells. Molecular and Cellular Biology. Vol. 4, No. 12, p:2929-2931.
3. Borovinskaya; M.A, Shoji; S., Fredrick; K., and Jamie H.D. Cate; J.H.D. 2008. Structural basis for hygromycin B inhibition of protein biosynthesis.RNA. ; 14(8): 1590–1599.
4. Palaniappan; N., Dhote; V., Ayers; S., Starosta; A., Wilson; D., and Reynolds; K., 2009. Biosynthesis of the Aminocyclitol Subunit of Hygromycin A in Streptomyces hygroscopicus NRRL 2388, Chemistry & Biology (2009), doi:10.1016/j.chembiol.2009.10.013
5. Pardo; J.M, Malpartida; F., Rico; M., and Jimene; A. , 1985. Biochemical Basis of Resistance to Hygromycin B in Streptomyces hygroscopicus - the Producing Organism. Journal of General Microbiology. 131, p:1289-1 298
6. Reid; G. 1999. The Scientific Basis for Probiotic Strains ofLactobacillus. Appl. Environ. Microbiol.vol. 65 no. 93763-3766.
7. Soltanmohammadi; B., Jalali-Javaran; M., Rajabi-Memari; H., and Mohebodini; M. 2014.Cloning, Transformation and Expression of Proinsulin Gene in Tomato (Lycopersicumesculentum Mill.).Jundishapur J Nat Pharm Prod. ; 9(1): 9–15.
8. Vickers; C.E, Bydder; S.F, Zhou; Y., and Nielsen; L.K. 2013. Dual gene expression cassette vectors with antibiotic selection markers for engineering in Saccharomyces cerevisiae. Microbial Cell Factories,12:96
9. Zalacain M, Malpartida F, Pulido D, Jiménez A. 1987. Cloning and expression in Escherichia coli of a hygromycin B phosphotransferase gene from Streptomyces hygroscopicus. Eur J Biochem., 15:162(2):413-8.