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ORIGINAL PAPER

Development of a pair of bifunctional expression vectors for *Escherichia coli* and *Bacillus licheniformis*

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Abstract A pair of bifunctional expression vectors, pBL-WZX and pHY-WZX, for *Escherichia coli* and *Bacillus licheniformis* was constructed to express interesting genes in a secretory manner. The vectors contain an expression cassette consisted of the promoter and signal peptide region of *B. licheniformis amyL* as well as an artificial multiple cloning site and a terminator and utilize kanamycin-resistance and/or tetracycline-resistance for selection in both *B. licheniformis* and *E. coli*. Both vectors contain a part of 3′ terminal fragment of *B. licheniformis amyL*. The 5′-terminal or 3′-terminal fragment of *B. licheniformis amyL* can cause the integration and amplification of expression cassette in the chromosome of *B. licheniformis* under a kanamycin-selection pressure. pBL-WZX is an integrational vector while pHY-WZX is free one for *B. licheniformis*. Both vectors were succeeded in secretory expression of *manL* in both *B. licheniformis* and *E. coli*.

Keywords Expression vector · *Bacillus licheniformis* · Promoter of *amyL*

Introduction

Bacillus licheniformis, a Gram-positive, spore-forming and facultative anaerobic bacterium widely distributed as a saprophytic organism in the environment, has numerous commercial and agricultural uses. The species has been used for decades in the manufacture of industrial enzymes including several proteases, α-amylase, penicillinase, pentosanase, cycloglucosyltransferase, β-mannanase and several pectinolytic enzymes [1, 7]. Specific *B. licheniformis* strains are also used to produce lipopeptide surfactants, peptide antibiotics such as bacitracin and proticin in addition to a number of specialty chemicals such as citric acid, inosine, inosinic acid and poly-γ-glutamic acid [2, 9]. These features illustrate the economic importance of the organism. Several attempts have been made to exploit these traits even more by improving production yields [6].

An interesting endeavor is to develop *B. licheniformis* as an efficient expression system for foreign genes since it can produce industrial exoenzymes with a prodigious yield up to 25 g protein per liter. Furthermore, the whole genome sequence has recently been disclosed by two different groups [13, 16], which additionally facilitates in vivo genetic manipulation in such industrial species. However, comparison to its closely related *B. subtilis*, *B. licheniformis* as host cells for expression of foreign genes as well as its associated genetic tools such as cloning and expression vectors for genetic modification and/or manipulation is still undeveloped [15].

In this article, we report the construction of a pair of bifunctional expression vectors for *E. coli* and *B. licheniformis*.

Materials and methods

Strains, plasmids and culture conditions

E. coli JM109 was used for cloning and expression experiments. *E. coli* cells were grown in LB medium

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[14] supplemented with antibiotics (at 50 μg/ml) when necessary. *B. licheniformis* CICIM B0204 and CICIM B2004 were purchased from the Culture and Information Center of Industrial Microorganisms of China Universities at Southern Yangtze University (http://www.cicim-cu.sytu.edu.cn). *B. licheniformis* B0204 [11], an alpha-amylase over-producer with no detectable activity of β-mannanase, was used as host cells or for cloning of *amyL*, a gene encoding a thermotolerant α-amylase. *B. licheniformis* B2004 [10] was used for cloning of *manL*, a gene encoding a mannanase. *B. licheniformis* cells were propagated at 37°C on LB medium supplemented with 7.5 μg/ml kanamycin when necessary. Plasmids used in this study are listed in Table 1.

Antibiotic minimum inhibitory concentrations

Growth of *B. licheniformis* B0204 was tested in LB medium in the presence of tetracycline (at 5, 10, 20, and 30 μg/ml) and kanamycin (at 2.5, 5, 7.5 and 10 μg/ml). Cell growth was followed by measuring absorbance at 660 nm.

Transformation

Transformation of *B. licheniformis* was performed by an electroporation protocol described by Xue et al. [17] with following modification. The electroporated mixture was recovered for 4 h at 37°C in LB containing 0.7 mol/l mannitol. The suspension was then spread on LB agar plates containing kanamycin (at 7.5 μg/ml) and cultivated for 2 days at 37°C.

DNA manipulations

DNA manipulations were performed using conventional techniques [14]. DNA was recovered from agarose gels with the QIAquick gel extraction kit (Qiagen, Germany). The nucleotide sequence was determined using a BigDye Terminator cycle-sequencing kit for ABI 3200 PRISM (Applied Biosystems).

Construction of plasmids

The plasmids pBL-WZX and pHY-WZX were constructed as Fig. 1. The 1.4 kb *amyL′* containing the promoter for *amyL* and the partial structural region of *amyL* was amplified with primers P1 (5′-AATGGATCCATTG GTAACTGTATCTCAGC-3′; underlined introduced *Bam*HI site) and P2 (5′-AACAGATCTGTTCAATTTT GTGTTTC-3′; underlined introduced *Bgl*II site) using *B. licheniformis* B0204 chromosomal DNA as template [11]. The 2.4 kb pBR322′ fragment containing the replication origin and ampicillin resistance cassette was amplified by inverse PCR with primers P3 (5′-AACAGATCTCTG CCTCGCGCGTTTCGGTGAT-3′; underlined introduced *Bgl*II site) and P4 (5′-TCGAGATCTCGAA TAA TAACTGTTATTTTTCA-3′; underlined introduced *Bgl*II site) using pBR322 DNA as template. *Bam*HI and *Bgl*II-digested *amyL′* was ligated with *Bgl*II-digested pBR322′ to yield plasmid pBR-*amyL′* (Fig. 1). The 3.2 kb fragment was amplified using pBR-*amyL′* as template by inverse PCR with primers P5 (5′-TTATAAGACGGGC AAAATAAAAAA ACGGATTTCCTTCAGGAAA TCCGTCCTCTCTGCTCTATCT AATTAGCATGC CATGGTACCCGG GAGCTCGAATTCTAGATTT GCCGCCGCTGCTGC-3′) and P6 (5′-TGGTCTTA TGACTTGGGCGCGCT-3′). During this step, a 700 bp structural region of *amyL* was removed, and a multiple cloning sites as well as an artificial terminator sequence were added just after the signal peptide region of *amyL* mediated by primer P5. This 3.2 kb fragment was purified and ligated with ΩKm cassette recovered from pSKsymΩKm [12] to yield plasmid pBL-WZX (Fig. 1).

To construct plasmid pHY-WZX, the multiple cloning site (MCS) of pHY300 PLK was removed by digestion with by *Eco*RI and *Hind*III followed the ends blunt with *Taq* DNA polymerase. The 1.8 kb

Table 1 Plasmids used in this study

Plasmid	Relevant characteristics	References
pBR322	Tet^R, Ap^R	[3]
pHY300 PLK	*E. coli-Bacillus* sp. shuttle vector; Tet^R, Ap^R	[8]
pSKsymΩKm	ΩKm in pSKsym	[12]
pBR-*amyL′*	*amyL′* in pBR322	This study
pBL-WZX	With an expression cassette under the control of P_{amyL}; Kan^R, Ap^R	This study
pHY-WZX	With an expression cassette under the control of P_{amyL}; Kan^R, Tet^R, Ap^R	This study
pBL-man	*manL* fused into the expression cassette of pBL-WZX	This study
pHY-man	*manL* fused into the expression cassette of pHY-WZX	This study

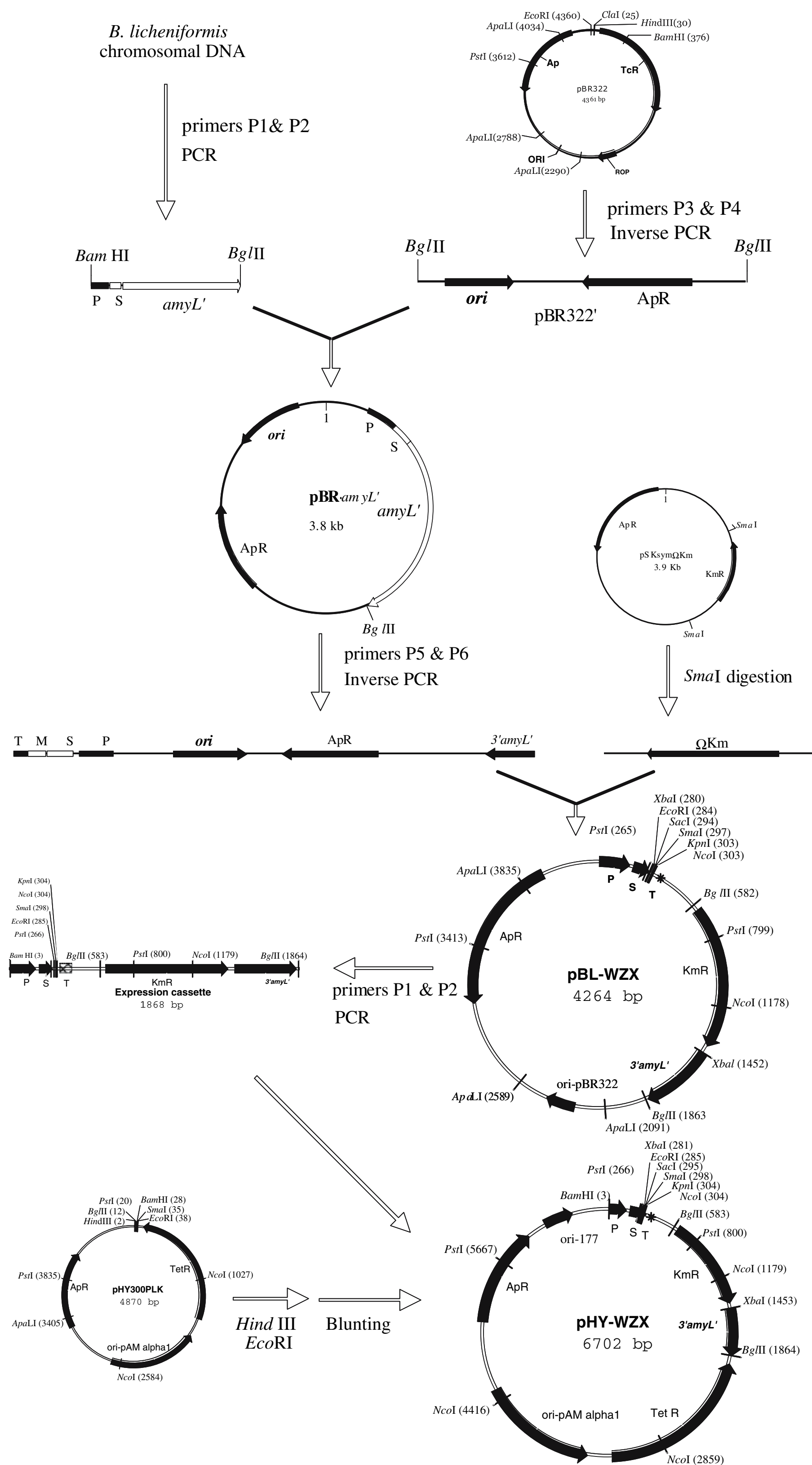

Fig. 1 Construction flowchart of pBL-WZX and pHY-WZX. *P* promoter of *B. licheniformis amyL*, *S* signal peptide region of *B. licheniformis amyL*, *T* artificial terminator with sequence of 5′-TAATTAGATAGAGCAGAGAGGACGGATTTCCTGAAGGAAATCCGTTTTTTTATTTTGCCCGTCTTATAA-3′, *M* multiple cloning site (*Xba*I, *Eco*RI, *Sac*1, *Sma*I, *Kpn*I, *Nco*I and *Sph*I) with sequence of 5′-GCAGCAGCGGCGGCAAATCTAGAATTCGAGCTCCCGGGTACCATGGCATGC-3′

expression cassette of pBL-WZX was amplified with primers P1 and P2 using pBL-WZX as template. The MCS-free pHY300 PLK fragment and the pBL-WZX expression cassette were subsequently co-ligated to yield plasmid pHY-WZX (Fig. 1).

Construction of recombinants and preparation of crude enzymes

Gene *manL* was used to examine the performance of pBL-WZX and pHY-WZX. The *manL* fragment encoding the mature β-mannanase was recovered from *B. licheniformis* B2004 chromosomal DNA by PCR with primers BLP1 (5′-ACGGAATTCCACACCGTTTC TCCGGTGAACC-3′; underlined introduced an *Eco*RI site) and BLP2 (5′-GGGCCTCTTATCGGCGGATTG GCTT-3′; underlined introduced a partial *Sma*I site). The 1.2 kb PCR product was digested with *Eco*RI and subsequently cloned in the *Eco*RI and *Sma*I sites of pBL-WZX or pHY-WZX to yield plasmid pBL-man or pHY-man.

The above constructed plasmids pBL-man and pHY-man were transferred into *E. coli* JM109 by calcium chloride method [14]. The resulting transformants were grown in LB medium at 37°C and 220 ×g for 18 h in 250 ml flasks with working volume of 30 ml. The cells were harvested and disrupted in a French Press. The supernatants was recovered by centrifugation at 13,000 rpm for 10 min and used as the crude enzymes for enzyme assays. The enzyme activity in the periplasmic space was determined according to Niu et al. [11]. Plasmids pBL-man and pHY-man were also electroporated into *B. licheniformis* B0204. The resulting transformants were grown in LB medium at 37°C and 250 rpm for 3 days in 250 ml flasks with working volume of 30 ml. The cells were removed by centrifugation and supernatants were collected as the crude enzyme for enzyme assays.

Enzyme and protein assays

To examine the activities of recombinant β-mannanase, the reaction mixture containing 1.5 ml of 0.5% locust bean gum (Sigma) in 0.1 M NaAc buffer (pH 5.8) and 0.1 ml of diluted enzyme solution was incubated at 65°C for 15 min. The amount of reducing sugars formed during the reaction was determined by the 3,5-dinitrosalicylic acid method [10]. One unit of β-mannanase activity was defined as the amount of enzyme that liberated 1 μmol equivalent D-mannose per minute. The protein concentration was determined by Bradford method [4] with bovine serum albumin fraction V (Sigma) as standard.

Plasmid stability

B. licheniformis B0204 cells harboring pHY-WZX were inoculated in LB medium without kanamycin and tetracycline and incubated at 37°C for 24 h and five generations. Culture samples were removed at 1 h intervals for numeration analysis. Total cell number was counted on LB-agar, and plasmid-containing cells were counted on LB-agar-kanamycin (7.5 μg/ml).

Results and discussion

Resistance of *B. licheniformis* B0204 to antibiotics

B. licheniformis B0204 grew well in the presence of 2.5 μg/ml kanamycin or 5 μg/ml tetracycline. No growth was detected in the presence of 5 μg/ml kamamycin or 25 μg/ml tetracycline. These results suggest that the corresponding resistance genes (i.e., Kan^R and Tet^R) could be used as selection markers on plasmids used to transform *B. licheniformis* B0204.

Construction of plasmids pBL-WZX and pHY-WZX

A pair of expression plasmids pBL-WZX and pHY-WZX were developed followed the method described in Fig. 1. Plasmid pBL-WZX and pHY-WZX are free plasmids in *E. coli*. Plasmid pHY-WZX is also a free plasmid whilst pBL-WZX is an integrated one in *B. lichenformis*. Both pBL-WZX and pHY-WZX can mediate the integration-amplification of an interesting gene in *B. lichenformis* chromosome through the 5′-*amyL*′ or 3′-*amyL*′ fragment franking the expression and selection cassettes and the integration-amplification can be selected or induced based on the ability of kanamycin resistance (Fig. 1). For the integration-amplification mediated by pHY-WZX, cells were grown at 50°C for 8 h to cure the free plasmid and force to integration before kanamycin selection pressure was applied. Both plasmids can introduce 2–8 multiple integration-amplification (data not shown).

Transformation by electroporation and plasmid stability

Transformation of the competent *B. subtilis* is convenient with efficiency of up to 10^4 per μg DNA [4, 17]. However, transformation of *B. licheniformis* with its competent cells is much more difficult since it lacks the competence stage [16]. On the other hand, electroporation has been used extensively to transform various *Actinobacillus*, *Bacillus, Haemophilus*, *Lactobacillus*,

Pasteurella, and *Pseudomonas* strains with sufficient efficiencies [5]. Our previous study [17] has optimized the electroporation conditions used for *B. subtilis* and *B. licheniformis*. We chose to use the electroporation conditions (i.e., 2.5 kV, 100 X, and 25 μF) used for *B. licheniformis* B0204. Electroporation of *B. licheniformis* B0204 with pHY-WZX gave an average of 1.2×10^2 CFU/μg plasmid. Significant difference was observed in the transformation yields using pHY-WZX DNA purified from between *E. coli* (i.e., 1.2×10^2 CFU/μg plasmid) and *B. subtilis* (i.e., 8.7×10^2 CFU/μg plasmid), suggesting that *B. licheniformis* has the restriction systems inhibiting transformation.

The stability of pHY-WZX and/or pBL-WZX was tested in *B. licheniformis* B0204 and in *E. coli* JM109. Plasmids pHY-WZX and pBL-WZX are much stable in *E. coli.* Plasmid pHY-WZX is stable in *B. licheniformis*, however, less stable than in *E. coli*. After five generations of growth in the absence of antibiotics, 99% of the *E. coli* cells still contained pHY-WZX, whereas 83% of the *B. licheniformis* cells did.

Expression of Tet^R and Km^R in *B. licheniformis*

To determine the efficiency of Km^R and Tet^R as selective markers in *B. licheniformis*, we tested their function once expressed from plasmid pHY-WZX or pBL-WZX. *B. licheniformis* B0204 harboring pHY-WZX grew on plates containing up to 15 μg/ml kanamycin or up to 50 μg/ml tetracycline. *B. licheniformis* B0204 integrating pBL-WZX grew on plates containing 5–35 μg/ml kanamycin. These results indicate that Tet^R and Km^R genes are functionally expressed in *B. licheniformis*. These results also suggest that Km^R gene can be used as a selective marker for the chromosomal integration of a foreign gene.

Expression of β-mannanase in *E. coli* and *B. licheniformis*

Two hybrid plasmids pBL-man and pHY-man were constructed by inserted *Eco*RI-digested *manL* into pBL-WZX and pHY-WZX. The pBL-man and pHY-man were transformed into *E. coli* JM109 and *B. licheniformis* B0204 yielding transformants JM109(pBL-man), JM109(pHY-man), B0204(pHY-man) and chromosome-integrated recombinant B0204(pBL-man), respectively. The β-mannanase activities expressed by the transformants were determined. Strains JM109(pBL-man) and JM109(pHY-man) showed 15–18 times more β-mannanase activity than *B. lichenformis* B2004 did [10]. Strain B0204(pHY-man) and B0204(pBL-man) did 5–7 times β-mannanase activity as *B. lichenformis* B2004 did [10]. Additionally, the signal peptide of *B. licheniformis* α-amylase by pBL-WZX leads 69.5% activity of β-mannanase to secret to the periplasmic space of *E. coli* [11].

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References

1. Berensmeier S, Singh SA, Meens J, Buchholz K (2004) Cloning of the *pelA* gene from *Bacillus licheniformis* 14 A and biochemical characterization of recombinant, thermostable, high-alkaline pectate lyase. Appl Microbiol Biotechnol 64:560–567
2. Birrer GA, Cromwick AM, Gross RA (1994) Gamma-poly(glutamic acid) formation by *Bacillus licheniformis* 9945 a: physiological and biochemical studies. Int J Biol Macromol 16:265–275
3. Bolivar F, Rodriguez RL, Greene PJ, Betlach MC, Heyneker HL, Boyer HW (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95–113
4. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
5. Brogan JM, Lally ET, Demuth DR (1996) Construction of pYGK, an *Actinobacillus actinomycetemcomitans–Escherichia coli* shuttle vector. Gene 169:141–142
6. Craynest M, Jorgensen S, Sarvas M, Kontinen VP (2003) Enhanced secretion of heterologous cyclodextrin glycosyltransferase by a mutant of *Bacillus licheniformis* defective in the D-alanylation of teichoic acids. Lett Appl Microbiol 37:75–80
7. Harwood CR (1992) *Bacillus subtilis* and its relatives: molecular biological and industrial workhorses. Trends Biotechnol 10:247–256
8. Ishiwa H, Shibahara H (1985) New shuttle vectors for *Escherichia coli* and *Bacillus subtilis*. Jpn J Genet 60:485–498
9. Kleinkauf H, von Döhren H (1987) Biosynthesis of peptide antibiotics. Annu Rev Microbiol 41:259–289
10. Niu D, Xu M, Ma J, Wang Z-X (2006) Cloning of the gene encoding a thermostable alpha-amylase from *Bacillus licheniformis* CICIM B0204 and functional identification of its promoter. Acta Microbiol Sin 46:576–580
11. Ma J, Yao T, Shi G-Y, Wang Z-X (2006) Purification and characterization of β-mannanase from *Bacillus licheniformis* strain 2004. J Food Sci Biotechnol 25(3):25–28, 32
12. Overhage J, Priefert H, Rabenhorst J, Steinbüchel A (1999) Biotransformation of eugenol to vanillin by a mutant of Pseudomonas sp. strain HR199 constructed by disruption of the vanillin dehydrogenase (*vdh*) gene. Appl Microbiol Biotechnol 52:820–828
13. Rey MW, Ramaiya P, Nelson BA, Brody-Karpin SD, Zaretsky EJ, Tang M, Lopez de Leon A, Xiang H, Gusti V, Clausen IG, Olsen PB, Rasmussen MD, Andersen JT, Jorgensen PL, Larsen TS, Sorokin A, Bolotin A, Lapidus A, Galleron N, Ehrlich SD, Berka RM (2004) Complete genome sequence of the industrial bacterium *Bacillus licheniformis* and comparisons with closely related *Bacillus* species. Genome Biol 5:R77
14. Sambrook J, Fritsch E., Maniatis T. (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

15. Tran LS, Szabo L, Orosz L, Sik T, Holczinger A (1998) Construction of a single-copy integration vector and its use to study gene expression in *Bacillus licheniformis*. Microbiology 144:2573–2578
16. Veith B, Herzberg C, Steckel S, Feesche J, Maurer KH, Ehrenreich P, Baumer S, Henne A, Liesegang H, Merkl R, Ehrenreich A, Gottschalk G (2004) The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. J Mol Microbiol Biotechnol 7(4):204–211
17. Xue M, Ma J, Wang Z-X (2004) Effect of high osmolarity on electrotransformation efficiency of bacteria. J Wuxi Uni Light Ind 23(4):98–100