Bacillus subtilis
Food Grade Expression System

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Contents

Introduction

Gram-positive bacteria are well known for their contributions to agricultural, medical, and food biotechnology, and for the production of recombinant proteins. Among them, ***Bacillus subtilis*** has been developed as an attractive host because of several reasons:

- It is non-pathogenic and is considered as a GRAS organism (generally regarded as safe), which means it can be used for food production;
- It has no significant bias in codon usage;
- It is capable of secreting functional extracellular proteins directly into the culture medium (at present, about 60 % of the commercially available enzymes are produced by *Bacillus* species);
- A large body of information concerning transcription, translation, protein folding and secretion mechanisms, genetic manipulation, and large-scale fermentation has been acquired, and the complete genome sequence of *B. subtilis* is available.

To increase the yield of heterologous proteins and to simplify operational processes, a wide variety of highly sophisticated plasmid-based expression systems have been developed. The here described ***Bacillus* Food Grade Expression System** was created to make the advantages of a Bacillus expression system also accessible to areas where antibiotic resistance gene markers are prohibited (e.g., food and feed industry). The ***Bacillus* Food Grade Expression System** enables stable vector-based large scale heterologous protein production by an alternative selection, without antibiotics.

The ***Bacillus* Food Grade Selection System** provides the following features:
- Stable high- or low-level expression without addition of any antibiotics
- All DNA contained in the final expression system is derived from *B. subtilis*
- No endotoxins are produced
- No inclusion bodies are formed
- Protease-deficient strain for producing secretory enzymes is available

The Food Grade Selection System

The ***Bacillus* Food Grade Selection System** is based on the interplay of an endogenous *Bacillus* **toxin EndoA** and its **antitoxin EndoB**.
EndoA (encoded by *ydcE*) is an endoribonuclease that specifically cleaves mRNA at a five Base U↓ACAU sequence (Pellegrini *et al*., 2005, Park *et al*., 2011). During normal growth conditions EndoA is inactivated by forming a heterohexameric complex with its cognate antitoxin EndoB (encoded by *ydcD*; Simanshu *et al*., 2013). Since the antitoxin is relatively unstable, it is essential for the cell to continuously produce sufficient amounts of EndoB to inactivate the more stable toxin. These characteristics are utilized for the here described selection system.

The mechanism of the selection system
The antitoxin encoding gene *ydcD* is located within the expression vector under the control of a constitutive *Bacillus* promoter whereas the toxin expression cassette is integrated into the genome and is controlled by a xylose-inducible promoter. If the expression of the toxin is induced by xylose but the plasmid with the encoded antitoxin gets lost, no more antitoxin

can be produced, leading to an active endoribonuclease that results in inhibition of protein synthesis, cell growth arrest, and finally dying of the cell.
This expression system has been proven to be very stable, with no plasmid loss observed in 100 generations. Compared with traditional antibiotic-dependent expression systems, this system results in greater biomass and higher titers of the desired products. This has been shown for the expression of the green fluorescent protein and the metabolic product hyaluronan, respectively (Yang *et al*., 2016).

The expression vectors
Dependent on the particular requirements there can be chosen between two *B. subtilis* expression vectors **pTTB1** and **pTTB2**. These vectors differ in their origin of replication and copy number concerning *B. subtilis*. **pTTB1** is a low copy number vector, replicating via theta replication modus, whereas **pTTB2** is high copy number, replicating by rolling circle mechanism.

Both vectors share the following features:
- For easier handling the vectors are designed as *B. subtilis* / *E. coli* shuttle vectors. The parts of the vector used for cloning with *E. coli* (*E. coli* origin ColE1 derivative and ampicillin resistance cassette Amp) can be eliminated afterwards by restriction enzyme cleavage and religation of the vector (for details see below). This technique connects the advantage of easy cloning (with *E. coli*) with the food grade property of *B. subtilis*.
- For food grade selection with *B. subtilis* the antitoxin-encoding gene *ydcD* is included under control of a constitutive *Bacillus* promoter (the strong P_{ylxM} promoter in pTTB1 and the weaker P_{aadD} promoter in pTTB2).
- A multiple cloning site for cloning the gene of interest downstream of the constitutive promoter P_{43} is provided.

The food grade *Bacillus* strains
For expressing the gene of interest under food grade conditions, two *B. subtilis* strains are available; TEA and WEA. Both strains contain the toxin expression cassette *ydcE* under control of the xylose-inducible promoter P_{xyl}, whereas the former *ydcDE* operon (containing the toxin and antitoxin gene) is deleted. The strain *B. subtilis* TEA is based on *B. subtilis* 168 Marburg and is recommended for intracellular protein expression and pathway engineering. *B. subtilis* WEA originated from the eightfold extracellular protease-deficient strain *B. subtilis* WB800N and is particularly constructed for secretory protein production. Both strains are suitable hosts for both the food grade low copy expression vector pTTB1 and the high copy expression vector pTTB2.

TEA: *trpC2 ydcDE*::P_{xyl}-*ycdE*

WEA: *nprE aprE epr bpr mpr::ble nprB::bsrΔvpr wprA::hyg cm::neo ydcDE*::P_{xyl}-*ycdE*; NeoR

Please note: this strain carries resistance to neomycin!

Storage and Handling Instructions

Storage and handling of plasmids
Plasmids are supplied lyophilized. Upon receipt, add 100 µl distilled water (final concentration 0.1 µg/µl) and incubate at 50 °C for 5 minutes. Vortex for 1 minute and store at -20 °C. Please note that all plasmids of this system are *E. coli* / *B. subtilis* shuttle vectors.

Storage and handling of *Bacillus* strains
The *Bacillus* strains are supplied as frozen cultures and shipped on dry ice. Store the stock at -80 °C. For propagation remove tube from freezer, scratch off some material from the surface of the frozen stock using a sterile loop. Streak onto an LB plate (see page 7), seal the plate with parafilm, and incubate at 37 °C overnight. *Bacillus* plates can be stored at 4 °C for 1 month. Use fresh bacteria, starting from one single colony for preparing competent cells.

Protocols

Growth conditions
Detailed protocols for *E. coli* and *B. subtilis* molecular genetic handling (growth, transformation, etc.) can be found in the relevant laboratory manuals such as Sambrook and Russell (2001).
B. subtilis and *E. coli* can be grown aerobically at 37 °C in 2xYT medium (Bagyan *et al*., 1998). Under optimal conditions the doubling time of *E. coli* is 20 min, of *B. subtilis* 30 min.
For selecting transformed *E. coli* cells, use 50-100 µg/ml ampicillin. For selecting transformed *Bacillus* TEA and WEA strains, add xylose to a final concentration of 2 g/L to the medium.

Vector propagation and cloning the DNA fragment of interest
Since both expression vectors (pTTB1 and pTTB2) are designed as *E. coli / B. subtilis* shuttle vectors, we recommend using *E. coli* for plasmid propagation and for cloning the DNA fragment of interest. Follow standard protocols for propagation in *E. coli*, *E. coli* plasmid mini preparation, restriction endonuclease cleavages, ligation of the desired DNA fragment into the vector, and transformation of *E.coli* (Sambrook and Russell, 2001). For selecting successfully transformed *E. coli* cells use 50-100 µg ampicillin. To retain food grade properties both vectors provide the opportunity to remove the functional *E. coli* parts (i.e., ampicillin resistance cassette and origin for replication) after finishing the construct. These parts can be removed by cleavage with one single restriction enzyme (for pTTB1 use SpeI, for pTTB2 use EcoRI) followed by purification of the larger fragment and religation. The religated vector does now only contain DNA that is derived exclusively from *B. subtilis* (with the exception of the cloned fragment).

Transformation of *Bacillus subtilis*

Preparation of competent *Bacillus subtilis* cells

The following protocol is adopted from Klein *et al*., 1992.

- Prepare an overnight culture in 5 ml HS medium at 37 °C under vigorous shaking. For inoculation we recommend using one single colony grown on an LB agar plate.
- Measure the OD_{600} (optical density at 600 nm) of the overnight culture and inoculate 50 ml HS medium to an OD_{600} of 0.05; incubate under vigorous shaking at 37 °C.
- Record the growth curve.
- **Immediately at transition of exponential to stationary growth phase** start taking samples of 10 ml, each 15 min
- Add 1 ml of sterile glycerol (87 %) to each sample, mix, and leave for 15 min on ice.
- Fractionate into 1 ml aliquots, freeze in liquid nitrogen, and store at -80 °C.
- Check one aliquot from each time point with a reference plasmid DNA (see below) to identify the time point(s) yielding high level competent cells; discard the non or low competent aliquots.

Transformation of competent *Bacillus subtilis* cells

- Thaw one aliquot at 37 °C
- Use these cells to inoculate 20 ml LS medium
- Shake cells slowly in a 30 °C water bath to obtain maximal competence (about 2 h)
- Take 1 ml aliquots into a glass tube or a 2 ml plastic reaction tube, add 10 µl of 0.1 M EGTA, and incubate for 5 min at room temperature
- Add about 1 µg plasmid DNA and incubate for 2 h at 37 °C while well shaking (well mixing is important when using plastic reaction tubes)
- If glass tubes were used, transfer cell suspension into a 2 ml plastic reaction tube
- Centrifuge, discard supernatant carefully, and resuspend the cells in the residual liquid remaining on the pellet
- Plate on selective 2xYT plates containing 0.2% xylose (see page 7)
- Incubate at 37 °C overnight

Media and Solutions

LB medium:	10 g tryptone 5 g yeast extract 5 g sodium chloride (NaCl) add distilled water to 1000 ml and autoclave
LB agar plates	add 1.5 % agar to the LB medium before autoclaving

2xYT medium:	16 g tryptone 10 g yeast extract 5 g sodium chloride (NaCl) add distilled water to 1000 ml and autoclave (121 °C, 15 min)
2xYT / xylose agar plates:	add 1.5 % agar to the 2YT medium before autoclaving, let cool down to 70 °C and add 2 % sterile-filtered xylose before pouring the plates
10x S-base (Spizizen's salt):	2 g $(NH_4)_2SO_4$ 14 g K_2HPO_4 6 g KH_2PO_4 1 g sodium citrate add distilled water to 100 ml and autoclave add 0.1 ml 1 M $MgSO_4$ after autoclaving
HS medium:	66.5 ml distilled water 10.0 ml 10x S-base 2.5 ml 20 % (w/v) glucose 5.0 ml 0.1 % (w/v) L-tryptophan 1.0 ml 2 % (w/v) casamino acids 5.0 ml 10 % (w/v) yeast extract (Difco) 10.0 ml 8 % (w/v) arginine, 0.4 % histidine autoclave all components separately tryptophan solution: sterile filtration
LS medium:	80.0 ml distilled water 10.0 ml 10x S-base 2.5 ml 20 % (w/v) glucose 0.5 ml 0.1 % (w/v) L-tryptophan 0.5 ml 2 % (w/v) casamino acids 5.0 ml 2 % (w/v) yeast extract (Difco) 0.25 ml 1 mM $MgCl_2$ 0.05 ml 1 mM $CaCl_2$ autoclave all components separately tryptophan solution: sterile filtration
0.1 M EGTA	dissolve 3.8 g EGTA in 50 ml distilled water adjust the pH to 7.2 using 10 N NaOH add distilled water to 100 ml; autoclave

References

Bagyan I. *et al*. (1998). The *katX* Gene, which codes for the catalase in spores of *Bacillus subtilis*, is a forespore-specific gene controlled by sigmaF, and KatX is essential for hydrogen peroxide resistance of the germinating spore; J Bacteriol. 180(8), 2057–2062

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Park *et al*. (2001). *Bacillus subtilis* MazF-bs (EndoA) is a UACAU-specific mRNA interferase; FEBS Lett. 585, 2526-2532

Pellegrini *et al*. (2005). The *Bacillus subtilis* ydcDE operon encodes an endoribonuclease of the MazF/Penk family and its inhibitor; Mol Microbiol. 56, 1139-1148

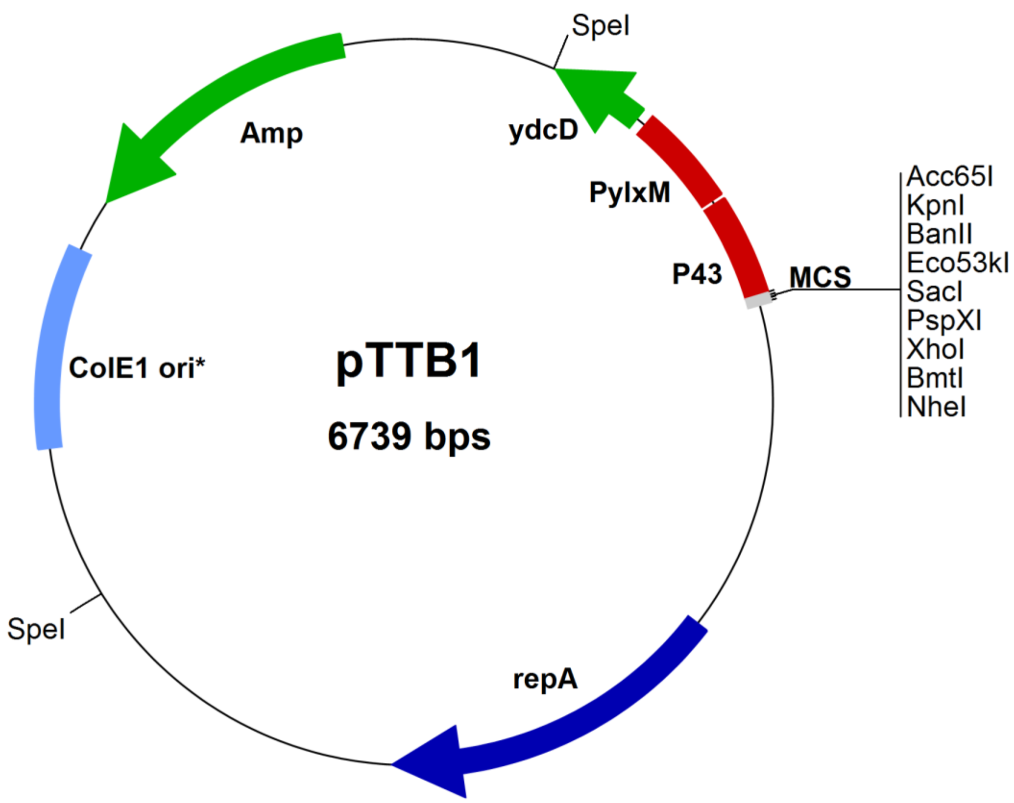
Sambrook, J. & Russell, D.W. (2001). Molecular cloning: a laboratory manual. (New York: Cold Spring Harbor Laboratory Press).

Simanshu D.K. *et al*. (2013). Structural basis of mRNA recognition and cleavage by toxin MazF and its regulation by antitoxin MazE in *Bacillus subtilis*; Mol.Cell 52, 447-458

Yang S. *et al*. (2016). Construction of a novel, stable, food-grade expression system by engineering the endogenous toxin-antitoxin system in *Bacillus subtilis*; J. Biotechnol. 219, 40-47

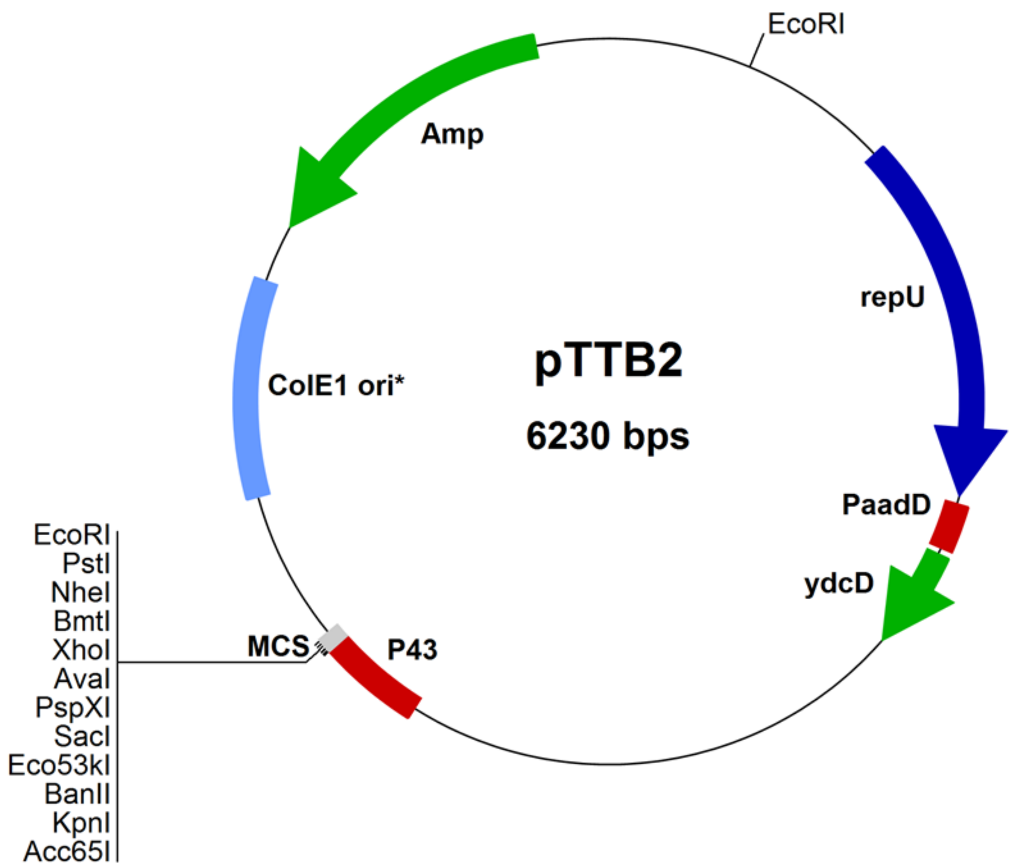
Vector Maps

Vector map pTTB1 (low copy)



	Type	Start	End	Name	Description
	Selectable genetic marker	724	443	ydcD	Antitoxin encoding gene
	Promoter	1054	755	PylxM	Strong constitutive promoter for expression of ydcD
	Promoter	1067	1365	P43	Constitutive promoter for expression of gene of interest
	MCS	1366	1395	MCS	Multiple Cloning Site
	Gene	2388	3422	repA	Gene of replication protein RepA (*B. subtilis*)
	Origin of replication	4918	5517	ColE1 ori*	origin of replication (*E. coli*); ColE1 incompatibility group
	Selectable genetic marker	6539	5679	Amp	Ampicillin resistance (*E. coli*)

Vector map pTTB2 (high copy)



	Type	Start	End	Name	Description
	Gene	814	1818	repU	Gene of replication protein RepU (*B. subtilis*)
	Promoter	1054	755	PaadD	constitutive promoter for expression of ydcD
	Selectable genetic marker	1987	2268	ydcD	Antitoxin encoding gene
	Promoter	3674	3947	P43	constitutive promoter for expression of gene of interest
	MCS	3948	3990	MCS	Multiple Cloning Site
	Origin of replication	4409	5008	ColE1 ori*	origin of replication (*E. coli*); ColE1 incompatibility group
	Selectable genetic marker	6030	5170	Amp	Ampicillin resistance (*E. coli*)

Please consider: EcoRI is no single cutter!

Quality Warranty

The vector features and restriction sites specified in this manual are verified by sequencing or checked for being functional. MoBiTec does not give any guarantee for sequence data of nonfunctional parts of the vectors.

Order Information, Shipping, and Storage

Order#	Product	Amount
PBS041	pTTB1	10 µg
PBS042	pTTB2	10 µg
Shipped at RT; plasmids are lyophilized from water and can be stored at 4 °C. Once the DNA has been dissolved in sterile buffer or water, store at -20 °C.		
PBS043	Bacillus subtilis strain TEA	1 ml
PBS044	Bacillus subtilis strain WEA	1 ml
Shipped on dry ice; store at -20 °C.		

The *Bacillus subtilis* Food Grade Expression System was developed by researchers of the Jiangnan University, China (Yang *et al*., 2016). All strains and vectors belonging to this system are for academic research and development only.

Commercial enterprises: To obtain strains and plasmids of this system, a license needs to be negotiated.