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# A Bacillus megaterium plasmid system for the production, export, and one-step purification of affinity-tagged heterologous levansucrase from growth medium

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## SUPPLEMENTAL MATERIAL

### Construction of plasmids

Molecular biology methods were outlined before (1-3). All constructed plasmids were amplified in *E. coli* DH10B and analysed by complete DNA sequence analysis of relevant regions by MWG Biotech (Ebersberg, Germany). They are listed in **table S1**. Synthetic oligonucleotides used are also listed in **table S2**. A detailed scheme for vector construction is depicted in **figure S1**.

For the insertion of the signal peptide encoding sequence into the *B. megaterium* expression plasmid pMM1520 (1), an additional unique restriction site (*Bsr*GI) had to be generated downstream of the xylose inducible promoter ($P_{xylA}$) of the *B. megaterium* expression plasmid pMM1520 (1). Primers QC1 and QC2 were used for the site-directed mutagenesis (QuikChange$^{TM}$ II, Stratagene, LaJolla, USA) of pMM1520 creating the vector pMM1522. This new restriction site provided the basis for the insertion of the signal peptide coding sequence consisting of three pairs of annealed synthetic oligonucleotides into the *Bsr*GI and *Bst*BI restriction sites of pMM1522. First 200 pmol of the oligonucleotides Oligo1 to Oligo6 (**Tab. S2**) were phosphorylated using T4 polynucleotide kinase. Pairwise matching oligonucleotides were combined, denatured for 3 min at 95 °C, hybridised for 1 min at the calculated optimal hybridization temperature, and finally incubated at 55 °C for 1 min. The now double stranded oligonucleotides were ligated to each other resulting in an 84 bp long oligonucleotide coding for $SP_{lipA}$. It was ligated into the *Bst*BI/*Bsr*GI digested vector pMM1522. The resulting vector encoding $SP_{lipA}$ but missing the *Bst*BI restriction site was named pMM1525.

For the construction of pSTOP1522 an *Age*I restriction site and a stop codon with a 3 bp downstream *Nru*I restriction site were successively introduced into pMM1522 using the site directed mutagenesis method QuikChange$^{TM}$ II and the primer pairs QC3/QC4 and QC5/QC6 (**Tab. S2**).

The DNA sequences for affinity tags were introduced into the expression vector pSTOP1522 using the synthetic oligonucleotide strategy as described. For the construction of expression vector pSTREP1522 oligonucleotides Oligo7 and Oligo8 (**Tab. S2**) encoding the Strep-tag II were hybridized and inserted into the *Spe*I/*Sac*I digested vector pSTOP1522. Expression

vector pHIS1522 was obtained by insertion of the hybridized oligonucleotides Oligo9 and Oligo10 (**Tab. S2**) encoding a $His_6$-tag into *Sph*I/*Age*I digested pSTOP1522.

During the construction of pMM1525 described above, the mutated plasmid pMM1533 with an additional *Bst*BI site downstream the DNA sequence encoding $SP_{lipA}$ was isolated. The *Bst*BI site of pMM1533 was now useful for inserting $SP_{lipA}$ upstream the Strep-tag II encoding sequence of pSTREP1522. In order to prepare the vector for the in frame insertion of target genes downstream of the affitiny tag DNA sequence, the *Sfo*I site of pMM1533 was eliminated by site directed mutagenesis using the primers QC7 and QC8 resulting in pADBm5. The DNA fragments encoding Strep-tag II and the vector were obtained by a *Xho*I and *Bst*BI digest of the plasmids pSTREP1522 and pADBm5, respectively. Both DNA fragments were combined and ligated in a way that the unique *Sfo*I site downstream of the Strep-tag II coding sequence was maintained. The resulting plasmid was named pSTREP1525. For the construction of pHIS1525, fragments from *Xho*I and *Bgl*II digested pMM1525 and pHIS1522 were combined and ligated. The affinity tags of pHIS1525 and pSTREP1525 were combined in one vector using the outlined strategy resulting in pSTREPHIS1525.

Next the part of the *L. reuteri* levansucrase gene, encoding an active and soluble protein in *E. coli,* was introduced into the various constructed vectors. The gene of LevΔ773MycHis including the N-terminal Myc-epitope and the $His_6$-tag coding sequence was amplified by PCR using primers PCR1 and PCR2 with pBAD-*lev* (4) as template. In order to obtain the original N-terminus of the mature levansucrase, the gene had to be cloned directly downstream of the signal peptide encoding sequence. For this purpose the gene was firstly inserted into another restriction site of the MCS, here the *Sac*I site. The employed primer PCR1 including the *Sac*I site for cloning carried an additional downstream *Sfo*I site, while a *Sph*I site was encoded in primer PCR2. Cloning of the *Sac*I/*Sph*I digested PCR fragment into the appropriately cut pMM1525 resulted in pMMBm6. The resulting preliminary plasmid contained a linker region between the coding sequence of the signal peptide and target protein. This linker contained two *Sfo*I sites – one in the sequence encoding the signal peptide cleavage site of pMM1525 and one inserted via the primer of the PCR product. This linker was quickly eliminated by *Sfo*I digestion and subsequent religation to obtain a plasmid encoding $SP_{lipA}$ directly followed downstream by the target gene resulting in plasmid pMMBm7. The vector encodes $SP_{lipA}$ directly upstream to the coding sequence of LevΔ773MycHis. A plasmid encoding the levansucrase without Myc-epitope and $His_6$-tag was constructed by insertion of a stop codon directly downstream the LevΔ773 coding

sequence by site directed mutagenesis using primers QC9 and QC10. The resulting plasmid pMGBm4 was identified due to the concomitant elimination of a *Bgl*II restriction site.

The gene of LevΔ773 without the coding sequence for the Myc-epitope and the $His_6$-tag was amplified by PCR using primers PCR3 and PCR4 with pMGBm4 as template. Primer PCR3 included a *Bgl*II restriction site at its 5'-end followed by two cytosines important for cloning of the target gene in the correct reading frame. A *Sph*I restriction site was part of primer PCR4. Cloning of the *Bgl*II/*Sph*I digested PCR fragment in the *Bgl*II/*Sph*I restricted pSTREP1525 and pSTREPHIS1525 resulted in pRBBm13 and pRBBm16, respectively. For pRBBm15 the gene of LevΔ773 without the coding sequence for the Myc-epitope and the $His_6$-tag was amplified by PCR using primers PCR1 and PCR4. Here, the same strategy was employed for construction of pMMBm7 from pMMBm6. Cloning of the *Sac*I/*Sph*I digested PCR fragment in the *Sac*I/*Sph*I cut pHIS1525 resulted in pRBBm14. The linker region between the coding sequences of the signal peptide and LevΔ773 of pRBBm14 was eliminated as described for pMMBm7 above to result in pRBBm15.

**Table S1**. Plasmids used in this study

| **Name** | **Description** | **Reference or source** |
|---|---|---|
| pMM1520 | Shuttle vector for cloning in *E. coli* (*Ap*$^r$) and expression under xylose control in *B. megaterium* (*Tc*$^r$) | Malten et al., 2005 |
| pMM1522 | *Bsr*GI restriction site inserted in pMM1520 upstream the start codon of the open reading frame (*orf*) with the multiple cloning site | this work |
| pSTOP1522 | pMM1522 derivative with stop codon directly downstream the *Nae*I restriction site followed by additional *Nru*I and *Age*I sites | this work |
| pMM1525 | pMM1522 derivative containing a DNA sequence coding for the signal peptide of the *B. megaterium* extracellular esterase LipA (SP$_{lipA}$) between the *Bsr*GI and *Bst*BI sites (**Fig. S2**) | this work |
| pMM1533 | pMM1525 with additional *BstB*I site downstream of SP$_{lipA}$ | this work |
| pADBm5 | pMM1533 derivative without *Sfo*I site downstream SP$_{lipA}$ | this work |
| pSTREP1522 | pSTOP1522 derivative – vector for the intracellular production of StrepII-tagged proteins in *B. megaterium* | this work |

| | | |
|---|---|---|
| pHIS1522 | pSTOP1522 derivative – vector for intracellular production of $His_6$-tagged proteins in *B. megaterium* | this work |
| pSTREP1525 | Vector for the secretion of recombinant Strep-tagged proteins in *B. megaterium* (**Fig. 2**); $P_{xylA}$-$SP_{lipA}$-Strep-Xa-MCS-STOP, $Tc^r$ (*B. megaterium*); $Ap^r$ (*E. coli*) | this work |
| pHIS1525 | Vector for the secretion of recombinant $His_6$-tagged proteins in *B. megaterium* (**Fig. 2**); $P_{xylA}$-$SP_{lipA}$-MCS-His-STOP, $Tc^r$ (*B. megaterium*); $Ap^r$ (*E. coli*) | this work |
| pSTREPHIS1525 | Vector for the secretion of recombinant Strep-$His_6$-tagged proteins in *B. megaterium* (**Fig. 2**); $P_{xylA}$-$SP_{lipA}$-Strep-MCS-His-STOP, $Tc^r$ (*B. megaterium*); $Ap^r$ (*E. coli*) | this work |
| pBAD-*lev* | pBAD/Myc-HIS C with *Lactobacillus reuteri* 121 levansucrase LevΔ773 gene | (4) |
| pMMBm6 | *levΔ773*MycHis cloned downstream $SP_{lipA}$ with linker in *Sac*I and *Sph*I sites of pMM1525 | this work |
| pMMBm7 | pMMBm6 after elimination of linker region creating $P_{xylA}$-$SP_{lipA}$-*levΔ773*MycHis | this work |
| pMGBm4 | pMMBm7 encoding LevΔ773 without Myc-epitope and $His_6$-tag | this study |

| | | |
|---|---|---|
| pRBBm13 | *levΔ773* cloned behind *strepII* in *Bgl*II and *Sph*I sites of pSTREP1525 creating $P_{xylA}$-$SP_{lipA}$-Strep*levΔ773* | this work |
| pRBBm14 | *levΔ773* cloned downstream $SP_{lipA}$ and linker in *Sac*I and *Sph*I sites of pHIS1525 | this work |
| pRBBm15 | pRBBm14 after elimination of linker region, creating $P_{xylA}$-$SP_{lipA}$-*levΔ773*His | this work |
| pRBBm16 | *levΔ773* cloned between *strepII* and $his_6$ in *Bgl*II and *Sph*I sites of pSTREPHIS1525 creating $P_{xylA}$-$SP_{lipA}$-Strep*levΔ773*His | this work |

**Table S2.** Primers used in this study.

| Name | Sequence 5'-3' |
|---|---|
| **QC1** | CAAAGGGGGAAA*TGtacA*ATGGTCCAAACTAGTTCG |
| **QC2** | GAACTAGTTTGGACCAT*TgtaCA*TTTCCCCCTTTG |
| **QC3** | CGATGCAAAACAAtaTCTGCAGCTGGTACC |
| **QC4** | GGTACCAGCTGCAGATtaTTGTTTTGCATCG |
| **QC5** | GCTAACGGATTC*ACCggT*CCAAGAATTGG |
| **QC6** | CCAAGCTTCTTGG*AccGGT*GAATCCGTTA |
| **QC7** | CGCATGCCGGCtaaACC*TCGCgA*ACGGATTCACCG |
| **QC8** | CGGTGAATCCGT*TcGCGA*GGTttaGCCGGCATGCG |
| **QC9** | CGTCTGGCGCAGGaGCCGCATTCGAAGATCTCC |
| **QC10** | GGAGATCTTCGAATGCGGCtCCTGCGCCAGACG |
| **Oligo1** | GTACAATGAAAAAAGTACTTATGGCATTCAT |
| **Oligo2** | TTACTTTTTTCATGAATACCGTAAGTAATAAAC |
| **Oligo3** | TATTTGTTTATCGCTGATTCTATCTGTTTTAG |
| **Oligo4** | AAATAGCGACTAAGATAGACAAAATCGGCG |
| **Oligo5** | CCGTCCGCCGTCCTGGCGCA*GGCGCC*GCATT |
| **Oligo6** | AGGCGGCAGACCGCGT*CCGCGG*CGTAAGC |
| **Oligo7** | CTAGTTTCGAATGGAGCCACCCGCAGTTTGAAAAAATCGAAGGGC GCCAGATCT |
| **Oligo8** | CCGGAGATCTGGCGCCCTTCGATTTTTTCAAACTGCGGGTGGCTCC ATTCGAAA |
| **Oligo9** | CCGGCCTGAGAGGATCGCATCACCATCACCATCACTAA |
| **Oligo10** | CCGGTTTAGTGATGGTGATGGTGATGCGATCCTCTCAGGCCGGCAT G |

**PCR1** TTATT*GAGCTC*T*GGCGCC*GATCAAGTAGAAAGTAACAATTACAACG

**PCR2** CAAGA*GCATGC*TGAAAATCTTCTCTCATC

**PCR3** TTATT*AGATCT*CCGATCAAGTAGAAAGTAACAATTACAACG

**PCR4** CAAGA*GCATGC*AATTGTTTTGCATCGGTATTCTTACC

Exchanged bases during site directed mutagenesis experiments are shown in lower case, while newly introduced restriction sites are depicted in *italic* letters.

**Table S3.** Protein sequence of levansucrase encoding plasmids.

| Secretion Vectors | | Protein Sequence |
|---|---|---|
| **Final** | **Encoded Protein** | |
| pMGBm4 | LevΔ773 | …AGADQV…AKQ |
| pRBBm15 | LevΔ773His | …AGADQV…AKQLHAGLRGS**HHHHHH** |
| pRBBm13 | StrepLevΔ773 | …AGAAFE**WSHPQFEK***IEGR*QISDQV…AKQ |
| pMMBm7 | LevΔ773MycHis | …AGADQV…AKQRSAAGTIWEFEAYVEQKLISEEDLNSAVD**HHHHHH** |
| pRBBm16 | StrepLevΔ773His | …AGAAFE**WSHPQFEK***IEGR*QISDQV…AKQLHAGLRGS**HHHHHH** |

The amino acid sequences are marked as follows: X for the signal peptidase cleavage site of the signal peptide LipA, **X** for the Strep-tag II, *X* for the Factor Xa cleavage site, **X** for the His$_6$-tag, X for the start and end of LevΔ773, X for the Myc-eptitope, X for the linker.

## Figure Legends

**Fig. S1.** Construction of expression plasmids for the production and secretion of tagged proteins of interest in *B. megaterium*. Elements for inducible gene expression in *B. megaterium* are the xylose inducible promoter and the gene for the xylose repressor (*xylR*). Elements for plasmid replication in *Bacillus sp*. are the origin of plasmid replication *oriU* and the *repU* gene. In addition, the plasmids contain a resistance to tetracycline (*tet*) as selection marker. Elements for plasmid replication in *E. coli* are the origin of replication *colE1* and the ampicillin resistance gene *bla*. The DNA coding for the $SP_{lipA}$ signal peptide, the Strep-tag II, the Factor Xa cleavage site and the $His_6$-tag are labeled. Only important unique restriction sites for the cloning are shown. For the cloning of genes of interest please see **figure 1**, where all restriction sites in the multiple cloning site (MCS) ranging from *Bgl*II to *Ngo*MIV are shown.

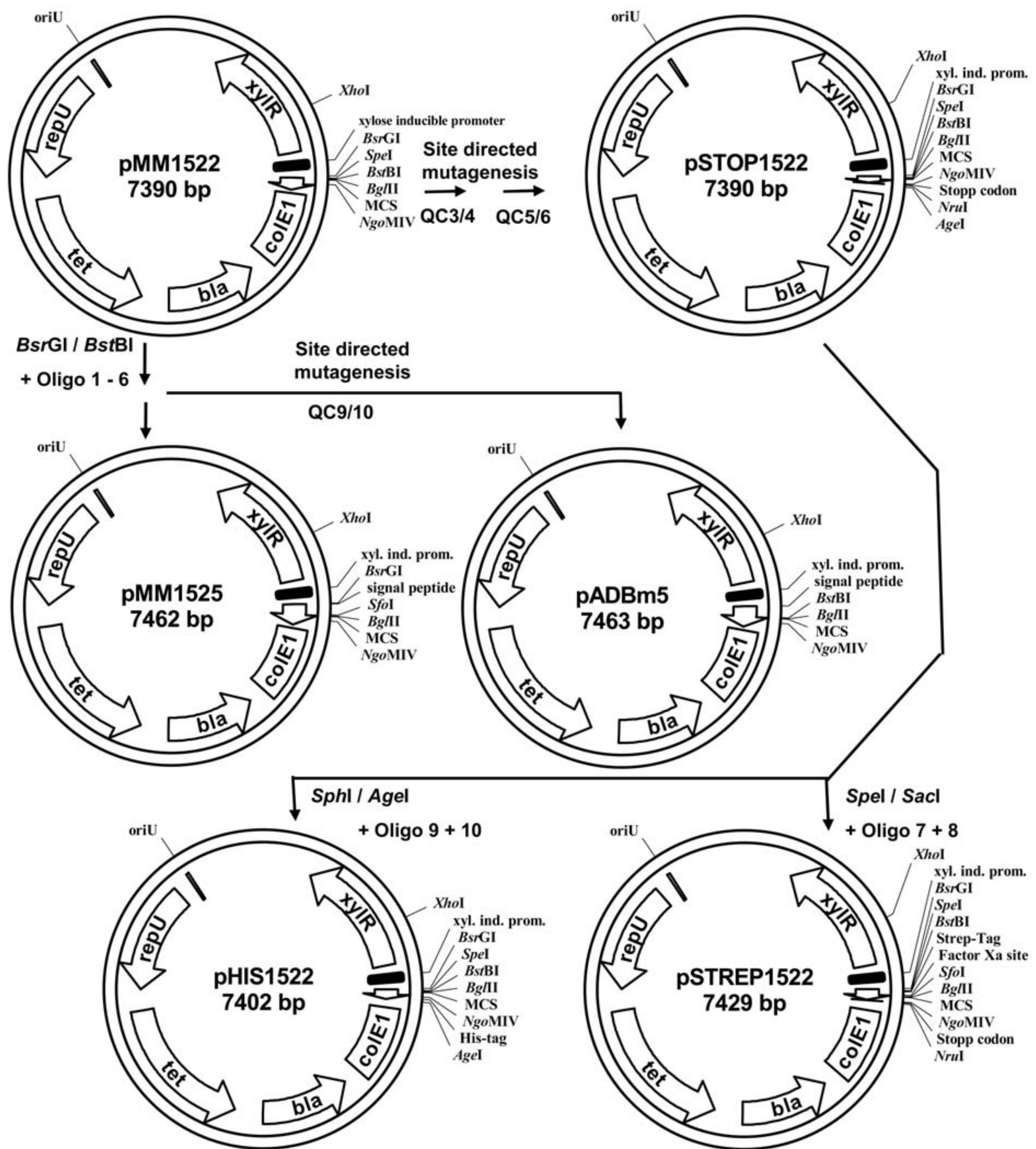


**Fig. S1 part A, Malten et al.**

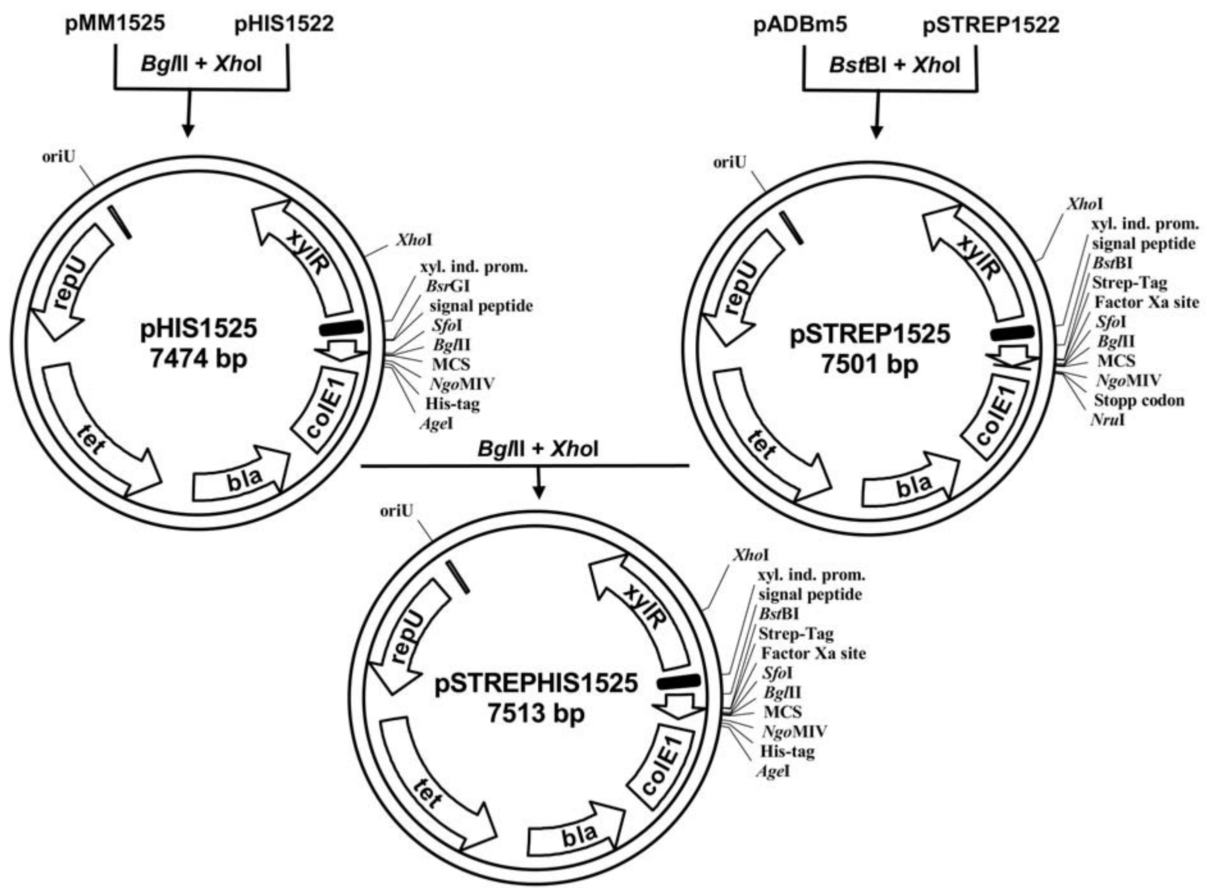


**Fig. S1 part B, Malten et al.**

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