Overproduction of *Bacillus amyloliquefaciens* extracellular glutamyl-endopeptidase as a result of ectopic multi-copy insertion of an efficiently-expressed *mpr* gene into the *Bacillus subtilis* chromosome

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Abstract

**Background:** Plasmid-less, engineered *Bacillus* strains have several advantages over plasmid-carrier variants. Specifically, their stability and potential ecological safety make them of use in industrial applications. As a rule, however, it is necessary to incorporate many copies of a key gene into a chromosome to achieve strain performance that is comparable to that of cells carrying multiple copies of a recombinant plasmid.

**Results:** A plasmid-less *B. subtilis* JE852-based strain secreting glutamyl-specific protease (GSP—the protein product of the *mpr* gene from *B. amyloliquefaciens*) was constructed that exhibits decreased levels of other extracellular proteases. Ten copies of an *mprB.amy* cassette in which the GSP gene was placed between the promoter of the *B. amyloliquefaciens* rplU-rpmA genes and the Rho-independent transcription terminator were ectopically inserted into designated (3 copies) and random (7 copies) points in the recipient chromosome. The resulting strain produced approximately 0.5 g/L of secreted GSP after bacterial cultivation in flasks with starch-containing media, and its performance was comparable to an analogous strain in which the *mprB.amy* cassette was carried on a multi-copy plasmid.

**Conclusion:** A novel strategy for ectopically integrating a cassette into multiple random locations in the *B. subtilis* chromosome was developed. This new method is based on the construction of DNA fragments in which the desired gene, marked by antibiotic resistance, is sandwiched between “front” and “back” portions of random chromosomal DNA restriction fragments. These fragments were subsequently inserted into the targeted sites of the chromosome using double-cross recombination. The construction of a marker-free strain was achieved by gene conversion between the integrated marked gene and a marker-less variant carried by plasmid DNA, which was later removed from the cells.

**Background**

Gram-positive bacteria are widely used for biotechnology applications, including vaccine delivery [1-3] and in situ production of anti-infective protectants [4] and microbicides [5]. These microorganisms serve as large-scale producers of nucleotides, vitamins, ribose, poly-γ-glutamic acids [6], absorbents [7], and insecticides [8]. *Bacillus* species are considered prospective cell-based factories for pharmaceutical proteins [9]. Currently, about 60% of commercially-available industrial enzymes are produced by selected and/or genetically-engineered *Bacillus* strains, most of which produce homologous proteins that are naturally secreted into the growth medium [6,9-15].

*Bacillus subtilis* produces numerous extracellular proteolytic enzymes. The alkaline serine protease subtilisin and the neutral protease (gene products of *aprE* and *nprE*, respectively) often constitute more than 90% of the total extracellular protease activity [9,16]. The
contribution of glutamic acid-specific protease (GSP) does not normally exceed 2% [17]. *B. subtilis* GSP, encoded by the *mpr* gene, is synthesized as an inactive pre-pro-peptide. This precursor is subsequently processed by the Sip and Bpr proteases, and mature extracellular GSP have a length of 220 amino acids [17]. Though they were initially subject of basic science investigation [18-20], some GSPs (from *B. licheniformis* in particular [21]) are now being utilized in commercial applications such as food production [22,23].

A traditional approach to the genetic engineering of *Bacillus* strains involves the introduction of multi-copy-number recombinant plasmids [10]. However, the construction of plasmid-less strains has recently become more relevant and practical. The preference for plasmid-less *Bacillus* strains is due to the genetic instability of many recombinant plasmids [24,25] and to official restrictions that concern the use of plasmid-carrying strains in large-scale industry in the First World [26]. Most often, the construction of plasmid-less *Bacillus* strains is performed by homologous recombination-mediated integration of the desired genes into the bacterial chromosome [10]. In some instances, specialized site-specific recombination [27] and transposition [28,29] are used for the same integrative purposes.

Recombination-mediated DNA incorporation can be implemented through either Campbell-type single-crossover integration of plasmids based on specialized vectors carrying DNA sequences homologous to the *Bacillus* chromosome or through the use of ectopic insertion, i.e. double-crossover recombination between the target in the chromosome and the homologous flanking sequences of the fragment of interest [10,30,31]. Both methods can be used for single-copy and multi-copy integrations [32-34]. Single-copy, plasmid-mediated integrants with inserted sequences bracketed by duplicated homologous regions are not stable under non-selective conditions due to the possible recombination-mediated elimination of the inserted plasmid [35]. Ectopic insertion(s) of a desired gene usually leads to significantly more stable recombinant strains. However, only a narrow set of well-characterized loci within the *B. subtilis* chromosome is normally used as targets for such insertions [10,36,37].

In this study, a recombinant, plasmid-less *B. subtilis* strain was developed that can efficiently produce and secrete GSP from *B. amyloliquefaciens*. Initially, three copies of the *mpr* gene were ectopically inserted into known *B. subtilis* genes encoding extracellular proteases. A novel, random integration methodology was then implemented to construct a stable strain with 10 *mpr* copies within the chromosome. Performance of the new strain was comparable to the strain carrying the *mpr* gene on a multi-copy plasmid, as exhibited by accumulation of the recombinant GSP in the media.

**Results**

**Cloning and expression of *B. amyloliquefaciens* mpr on a *B. subtilis* plasmid**

The nucleotide sequence of the *mpr* gene from *B. amyloliquefaciens* A-50 was not known. Primers for the amplification of *mpr* by PCR, *mpr-F/R* (the structures of the primers used in this study were presented in Additional file 1, Table S1), were therefore designed based on the available *B. amyloliquefaciens* ZB42 genome sequence (GenBank/EMBL accession number NC_009725) [38]. DNA amplicons of 972 base pairs (bp) in length were obtained and sequenced (GenBank accession number GU992366). The corresponding DNA sequence closely coincided with the *mpr*-containing sequence from *B. amyloliquefaciens* FZB42 (91% of identity) and covered the 909-bp open reading frame. An extended AG-rich block, including a *B. subtilis* Shine-Dalgarno sequence, AAGGAGG [39], was found upstream of the ATG codon of this ORF. The protein-coding ORF possessed 68% identity to well-characterized pre-pro-ORF from *B. subtilis* [17,18,20].

The *mpr*-carrier amplicon, flanked by artificial BgIII sites (P1-bmp5 and P2-bmp2 were used as the primers), was cloned into the BgIII site of the pHEA323 plasmid [40]. This placed it under the transcriptional control of the promoter (P_{rp}) of the rplU-rpmA genes from *B. amyloliquefaciens* A-50, which encode the L21 and L27 ribosomal proteins. In the resulting pHE52mpr recombinant plasmid, the cloned *mpr* gene became the central part of an artificial operon that was terminated by the Rho-independent transcription terminator (Ter) from the *pheA* gene of *B. amyloliquefaciens* A-50 (Figure 1). As was shown previously [40] and confirmed in the present study, the presence of Ter for the termination of efficient P_{rp}-mediated transcription is conducive to the stable inheritance of pHEA323 and its derivatives (i.e., *mpr*<sup>⁎</sup>*amy* cassettes (P<sub>rp</sub>*mpr*-Ter) in the pHE52mpr plasmid and/or integrated into the bacterial chromosome.

*Mpr* expression studies were performed with the *B. subtilis* strain JE852 serving as a recipient. This strain was a double mutant for genes encoding two major extracellular proteases (*mpr*E512, aprE851), which simplified the assessment of recombinant GSP activity.

Initially, a plasmid-carrying, recombinant GSP-producing strain was constructed via the transformation of *B. subtilis* JE852 with pHE52mpr. The level of GSP accumulation was analyzed by the semi-quantitative skim milk method on media containing different carbon sources and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of extracellular
proteins. It was shown in these experiments that the expression of the mprB.amy cassette was under carbon catabolite control (CCC) in B. subtilis. Indeed, when glucose or maltose were added to the media, B. subtilis JE852/pHE52 grew well but did not form clear, hydrolytic zones around colonies on milk agar. On the other hand, during growth on medium containing soluble starch as the sole carbon source, abundant amounts of GSP accumulated and were easily distinguished from the other extracellular proteins by SDS-PAGE. The main mechanism of CCC in Bacillus has been well studied [41-44]. CCC is implemented through the binding of the CcpA-mediated regulatory protein complex to special DNA sites known as catabolite responsive elements (cre). This binding causes carbon catabolite repression (CCR) or activation (CCA), depending on the position of the cre. For example, when the regulatory complex binds to cre that is located downstream of the transcription initiation point, it evokes a transcription roadblock that leads to CCR of the corresponding genes [44]. Two putative catabolite responsive elements that were homologous to the known [45-47] consensus sequence are also indicated.

CCR and that there may be changes in the control of enzyme biosynthesis, secretion, and/or maturation at different stages of bacterial growth [41,42,47].

Defining the mechanism of CCR modulation of GSP extracellular accumulation was outside the scope of the present paper. We showed that GSP production was significantly increased during fermentation of the B. subtilis JE852/pHE52mpr strain on TYS6C media, in which starch was the main carbon source. In this media, an enhanced biomass (growing up to an OD600 of around 40-50) and high level of extracellular GSP accumulation (up to approximately 0.5 g/L, as semi-quantitatively determined by SDS-PAGE, see Materials and methods) were detected. These results were obtained for the strain carrying multi-copy-number recombinant plasmids, suggesting that the integration of multiple copies of the mprB.amy-cassettes into the bacterial chromosome is indispensable for achieving comparably high GSP production levels in a plasmid-less Bacillus strain.

Ectopic insertion of mprB.amy cassettes into genes encoding known extracellular proteases

Ectopic insertion of several mprB.amy cassettes was performed to simultaneously inactivate known extracellular protease genes of B. subtilis: aprE, epr and nprB. The overall scheme of mprB.amy cassette insertion had three stages (see Figure 2 where the mprB.amy cassette insertion into the aprE851 allele of B. subtilis JE852 strain is shown as an example). First, a linear DNA fragment consisting of an antibiotic resistance (AntR) marker flanked with homologous arms was integrated into the corresponding chromosomal region via double-crossover recombination. Then, the AntR marker was exchanged for the mprB.amy cassette by gene conversion (for a review, see [48,49]) between the chromosome and the

Figure 1 Structure of the pHE52mpr plasmid carrying the mprB.amy cassette. Pmp - promoter of the rplU-rpmA genes from B. amyloliquefaciens A-50; Ter - Rho-independent transcription terminator of the pheA gene. The BglII-site-ended (boldface) followed by 5'-portion of B. amyloliquefaciens A-50 mpr gene is shown. In this part, SD-sequence and ATG-codon are marked as boldface and capital letters, respectively. Two putative cre elements, which are homologous to the known [45-47] consensus sequence, are also indicated.

Figure 2 Scheme of the targeted mprB.amy-cassette integration into the JE852 chromosome (aprE851 is shown as an example of the target gene).
autonomously replicating \textit{mpr}^{B.amy}-carrying plasmid, and this was followed by plasmid curing and construction of the plasmid-less, targeted integrant.

The \textit{aprE851} gene in \textit{B. subtilis} JE852 was chosen as the first target gene for \textit{mpr}^{B.amy} cassette insertion, primarily to prevent reversion of the mutant allele to the wild-type phenotype during the proposed long-term construction of a GSP-producing, plasmid-less strain. The \textit{Cm}^R gene from pC194 [50] was used as the AntR marker for selective integration at the first stage. The linear fragment, used for \textit{aprE851} gene disruption, was constructed \textit{in vitro} by overlapping PCR technique (see \textbf{Materials and methods} and Additional file 2, Figure S1 for details). The targeted integration of the \textit{Cm}^R marker was followed by gene conversion using the \textit{Em}^R-marked recombinant plasmid pCBT(yhfO-\textit{mpr}^{B.amy}-yhfN) and subsequent selection of the obtained \textit{Em}^R \textit{Cm}^S clones, which were generated at a frequency of around 2%. Finally, the plasmid-less (\textit{Em}^S) variants were selected after bacterial cultivation in liquid erythromycin-free medium. All integration stages were assessed by PCR, and the chromosome structure of the \textit{B. subtilis} JE852\textit{aprE851::mpr}^{B.amy} strain was analyzed by PCR and using Southern hybridization.

The same method, with modifications based on the nucleotide sequences of the target genes, was used for step-by-step ectopic insertion of the \textit{mpr}^{B.amy} cassette into the \textit{epr} and \textit{nprB} genes, encoding two minor extracellular proteases of \textit{B. subtilis} (see \textbf{Materials and methods} and Additional file 1, Table S1 for details). This process resulted in the desired \textit{B. subtilis} strain, a JE852-based plasmid-less, marker-less strain, JE852 (\textit{aprE851}, \textit{epr}, \textit{nprB}::\textit{mpr}^{B.amy}, with three integrated \textit{mpr}^{B.amy} cassettes.

The dependence of GSP accumulation on the integrated \textit{mpr}^{B.amy} copy-number (N) was evaluated according to the semi-quantitative plate test based on casein hydrolysis (Figure 3) and using SDS-PAGE analysis of extracellular bacterial proteins (Figure 4). The results showed that GSP production was significantly lower than that of a recombinant strain that had multiple plasmid copies, even for the plasmid-less strain, which had three cassette insertions (N = 3). This finding suggested that the process of cassette amplification needed to be continued. However, simplifying the procedure to obtain many single-copy integrants and then combining the variants possessing segregation stability became an attractive option.

\textbf{Integration of the \textit{mpr}^{B.amy} cassette into random sites in the bacterial genome}

A key aspect of the novel strategy presented here is the construction of DNA fragments in which the AntR-marked cassette (\textit{mpr}^{B.amy}-AntR) is sandwiched between the “front” and “back” portions of randomly digested fragments of the recipient chromosome. The proposed scheme is presented in Figure 5. Initially, pHE52(\textit{mpr-Cm}^R) was constructed (see \textbf{Materials and methods}). This plasmid carried the \textit{mpr}^{B.amy} \textit{Cm}^R cassette that was bracketed by \textit{PstI}-sites and did not contain internal \textit{BamHI}-sites. The \textit{PstI}-generated \textit{mpr}^{B.amy} \textit{Cm}^R cassette is marked as (\textit{a}) in Figure 5. The \textit{BamHI}-generated DNA fragments of the \textit{B. subtilis} JE852 chromosome (\textit{b}) fragments in Figure 5) were self-circularized by T4 ligase at a low DNA concentration and subsequently cleaved by \textit{PstI}. (\textit{b})-fragments in Figure 5 were a mixture of \textit{PstI}-site(s)-carrying (\textit{b1}) and \textit{PstI}-site-free (\textit{b2}) fragments. The (\textit{b1})-fragment with two internal \textit{PstI}-sites was shown in the Figure 5 for simplicity. The self-circularized (\textit{b2}) fragments could not be linearized by \textit{PstI} and so would not be later linked with the (\textit{a})-fragment. In contrast, the self-circularized (\textit{b1})-fragments hydrolyzed by \textit{PstI} generated a mixture of \textit{BamHI}-site-carrier (\textit{c1}) and \textit{BamHI}-site-free (\textit{c2}) linear DNA fragments. The ligation of (\textit{c1})-fragments with (\textit{a})-fragment followed by \textit{BamHI} treatment caused formation of linear (\textit{Lin}) fragments consisting of the cassette of interest sandwiched by “front” and “back” homologous arms. These (\textit{Lin})-fragments could participate in subsequent double-cross recombination-mediated integration into the bacterial chromosome. (\textit{c2})-fragments could be
ligated with the (a)-fragment as well. These circular, recombinant DNAs, (Cir)-fragments, were resistant to BamHI-mediated cleavage and could be integrated into the chromosome only via single-cross Campbell-type recombination. It could be supposed that the number of (Cir)-mediated integrants would be more than (Lin)-mediated ectopic insertions [10]. At the same time, the (Cir)-mediated integrant with the cassette sandwiched between directly repeated (c2)-fragments (see Figure 5) could be rather unstable due to the possibility of recombination-dependent elimination of the cassette.

The success of the strategy led to the formation of about 250 CmR clones after transformation of the \( B. \) subtilis JE852 strain. These colonies were then tested for their segregation stability (see Materials and methods for details). Fifteen transformants that demonstrated 100% segregation stability after 60 generations were used in the experiments that followed. According to data from the literature [10], it could be supposed that the stable integrants were obtained due to the intrinsic ectopic insertions, whereas transformants that manifested decreased segregation stability were the result of Campbell-type integration.

According to experimental evaluation (including growth on skim milk plates and SDS-PAGE analysis of extracellular proteins), all 15 stable integrants produced and secreted GSP at slightly variable levels, and the levels corresponded to the presence of one \( mpr^{B.amy} \)-CmR cassette in the chromosome of \( B. \) subtilis [17].
possessed three cassettes, *B. subtilis* JE852(*aprE851, epr, nprB*: *mprB.amy*). For cassette amplification, extracted chromosomal DNA from one CmR strain was used for transformation of a marker-less strain carrying C N copies of the mprB.amy-cassette (initially N = 3 in these experiments). Selection of CmR transformants led to the creation of a *B. subtilis* genome that contained N+1 copies of the gene encoding GSP. At the last step of this round of cassette amplification, the strain was rendered marker-less by gene conversion with pHE52mpr followed by plasmid curing. Then, the next CmR-marked cassette was inserted into the chromosome of the newly obtained strain, which carried N+1 cassettes.

The level of GSP secretion and the presence of all previously integrated cassettes in the bacterial genome were assessed at each stage of the cassette amplification process using Southern hybridization and SDS-PAGE analysis of extracellular proteins (Figure 4). As a rule, each subsequent generation displayed a slightly increased level of GSP accumulation in comparison to the previous generation, maintained the earlier integrated cassettes at their original positions in the bacterial genome and presented one novel hybridized DNA fragment that could be detected in the marker-less derivative of the corresponding donor strain.

Ultimately, a plasmid-less and marker-less strain carrying 10 copies of the mprB.amy cassette was obtained. This strain efficiently secreted GSP at the same level as the control, *B. subtilis* JE852/pHE52mpr.

**Conclusion**

Efficient production and secretion of *B. amylophilum* A-50 GSP by a recombinant plasmid-less *B. subtilis* strain was obtained. The mutant *B. subtilis* JE852 (*nprE, aprE*), which possessed significantly decreased levels of major extracellular proteases, was utilized as the initial recipient strain. The mprB.amy cassette, in which transcription of the mpr gene was controlled through a promoter that drives genes for ribosomal proteins in combination with a Rho-independent terminator, was expressed and stably maintained. Finally, the mprB.amy cassette was amplified by multiple ectopic insertions of the construct into the *B. subtilis* chromosome within known genes initially and then in random loci according to the methodology described above. The methods used for these insertions differed slightly but used the following general steps: (i) an AntR-marked linear DNA fragment was sandwiched between two arms that were homologous to a target in the bacterial genome, (ii) this fragment was incorporated using double homologous recombination, (iii) the marker was removed by gene conversion between the chromosome and an introduced plasmid and (iv) the incoming plasmid was eliminated from the cell.

It should be mentioned that the efficiency of iterative gene conversions using the same plasmid, pHE52mpr, decreased slightly with an increase in the copy number of the integrated cassettes. This efficiency was about (3 to 4)% when N = 3 or 4 but did not exceed 1% for strains with N = 9 or 10. Excisal markers that can be efficiently removed by different site-specific recombination events [27,51] might be preferable for the amplification procedure.

The constructed plasmid-less strain, which has 10 chromosomal mprB.amy cassettes, displayed essentially the same GSP production level as the recombinant plasmid-carrying strain. According to data in the literature, there are likely 20-30 copies of the pSM19035 replication-based plasmid in the recombinant plasmid-carrier strain [10,52,53]. This apparent incongruity has several explanations. First, the copy number of the recombinant mprB.amy-carrier plasmid could be lower than that of the vector, in particular, because of interference between plasmid replication and efficient intra-plasmid transcription. Second, expression levels of the same gene located in the chromosome vs. located on a plasmid could differ due to changes in the DNA curvature; dependence on restrained superhelical density is typical of protein-bound DNA molecules [54]. Third, Prp-mediated transcription of even ten copies of the mpr gene may be inherently efficient, such that the saturated translation/secretion machinery becomes the true bottleneck for extracellular GSP accumulation.

Segregation stability is a major factor that must be considered in the potential practical application of plasmid-less recombinant strains. As mentioned previously, only 10% of the clones that had a single-copy of the mprB.amy-carrying cassette integrated at random points within the bacterial chromosome possessed strong segregation stability. Amplification of the same cassettes in one genome could certainly decrease the strain’s stability due to the potential for homologous intrachromosomal recombination. Recombination between directly repeated cassettes can lead to internal chromosomal deletions such that the strains, possessing essential genes in regions between the cassettes, have to be protected from these genomic rearrangements. In turn, recombination between inversely repeated cassettes leading to chromosomal inversions [55,56] could be the basis of strain instability and, in particular, the decreased performance of the corresponding strain.

It seems useful to determine the integration points to finalize the construction of a set of stably-maintained single-copy cassette integrants. This determination could be performed using inverse PCR-based methods [57]. In this case, a task-oriented amplification of the cassettes could be performed to exclude the formation
of inverted repeats and to localize essential genes between directly repeated cassettes.

It is possible that this strategy of ectopic multi-copy integration would be helpful for the construction of a broad range of plasmid-less, marker-less, recombinant Bacillus strains for microbial technology applications.

Materials and methods

Bacterial strains, plasmids, and culture conditions

Strains and plasmids used in the present study are shown in Additional file 3, Table S2. Cells of B. amyloliquefaciens and B. subtilis were grown at 37°C in liquid LB media or LB with agar [58] supplemented by antibiotics (chloramphenicol (Cm, 5-10 mg/L) or erythromycin (Em, 10 mg/L) when necessary.

Cells were plated on skim milk (20%) test plates for semi-quantitative detection of the total extracellular protease activity; activity was determined by the size of the clearance zone around each colony [17,22].

The fermentation media TYS6C that was used for GSP production was composed of the following: 2% tryptone, 3% yeast extract, 6% soluble starch, 2% corn steep liquor (CSL), 0.1% CaCl₂ (added after autoclaving), and 1% CaCO₃ (added after sterilization) at pH 7.0. A final concentration of 10 mg/L Em was added to the media for cultivation of the plasmid-carrying strain. B. subtilis strains were cultured for 48 hours on a rotary shaker (at 220 rpm) at 37°C in 750-mL flasks containing 30 mL of media. Seed cultures were standardized by the preparation of freezer stock (~70°C) cultures in 20% glycerol. Then, 0.15 mL of the seed culture from the glycerol stock was used to inoculate 30 mL of TYS6C media for cultivation of the plasmid-carrying strain. B. subtilis strains were cultured for 48 hours on a rotary shaker (at 220 rpm) at 37°C in 750-mL flasks containing 30 mL of media. Seed cultures were standardized by the preparation of freezer stock (~70°C) cultures in 20% glycerol. Then, 0.15 mL of the seed culture from the glycerol stock was used to inoculate 30 mL of TYS6C media in a single 750-mL flask. Samples for SDS-PAGE were taken after 48 hours of bacterial cultivation.

TYS6 media was the same as TYS6 media, but without the CSL component. TYS6 media with 2%-4% glucose or maltose was used as the test media for subsequent integration.

Standard genetic engineering methods

Transformation of B. subtilis was performed using the method described by Spizizen [59].

Transformation of recombinant DNA and Southern hybridization were carried out in accordance with conventional protocols [60]. Chromosomal DNA of B. subtilis strains was hydrolyzed by EcoRI overnight, separated by electrophoresis in agarose and hybridized with biotinylated, mpr-containing PCR fragments that were amplified with mprFmprR primers using pHE52mpr as a template. The Biotin DecaLabel™ Kit and Biotin Chromogenic Detection Kits (Fermentas, Lithuania) were used to label and detect DNA.

Preparations of restriction enzymes, T4 DNA ligase and DNA polymerase I Klenow fragments from Fermentas were used. Taq DNA polymerase (Fermentas) or AccuTaqLA DNA polymerase (Sigma, USA) were used for PCR in accordance with the manufacturers’ instructions. The structures of all primers used in the present study are listed in Additional file 1, Table S1.

Construction of the pHE52mpr and pHE52(mpr-CmR) plasmids

The pairs of primers mprFmprR and P1-bmp5/P2-bmp2 were used for PCR-mediated amplification and then for cloning of the mpr gene from the chromosomal DNA of B. amyloliquefaciens A-50. The amplicons, generated in PCR with P1-bmp5/P2-bmp2 as the primers, were treated with BglII and inserted into the BglII site of the pHAE323 plasmid [40] to form the pHE52mpr plasmid. The CmR gene from the pC194 plasmid [50] was cloned into a BglII-site of the pHE52mpr plasmid located just downstream of the mpr gene (with coordinate (2,174), in Figure 1). As a result, the pHE52(mpr-CmR) plasmid carrying the mprB.amy-CmR cassette was obtained. The mprB.amy and mprB.amy-CmR cassettes had the mutual DNA fragments not only in proximal part, but in distal part, as well. The later included B. amyloliquefaciens DNA fragment of the pHE52mpr plasmid (about 1,800 bp in length) consisted of pheA gene and Ter. So, pHE52mpr plasmid could be efficiently used for gene conversion resulting in substitution of mprB.amy-CmR cassette integrated in the chromosome by the marker-less mprB.amy-cassette from the plasmid (see below).

Construction of the JE852(aprE851, epr, nprB):mprB.amy strain

The strain JE852(aprE851, epr, nprB):mprB.amy was constructed via step-by-step ectopic integration of three copies of the mprB.amy cassette into the aprE851, epr and nprB genes of the JE852 strain. For each integration, two target-specific DNA molecules were constructed: (i) linear CmR-carrier DNA fragments for the target gene inactivation and (ii) mprB.amy-carrier plasmids for gene conversion.

As for integration into the aprE851 gene, the linear DNA fragment, yhfO-CmR-yhfN, was constructed in vitro by overlapping PCR, as shown in Additional file 2, Figure S1. The final DNA ampiclon was treated with EcoRI and cloned into a pCB20-based [52] plasmid for the construction of pCBT(yhfO-CmR-yhfN). The later recombinant plasmid was used as a vector for the in vitro substitution of the CmR-marker by the Psrl-generated mprB.amy-cassette from pHE52mpr (Figure 1). The obtained pCBT(yhfO-mprB.amy-yhfN) plasmid was used for in vivo gene conversion, which resulted in construction of the JE852(aprE851):mprB.amy strain (Figure 2).

A linear DNA fragment for integration into the epr gene was designed using Pr7Pr8 as the primers for
PCR-mediated amplification of the *B. subtilis* 168 chromosome. Insertion of the *PstI*-generated amplicon with the Cm\(^R\) gene from pC194 (the primers-Pr9/Pr10) was between two *PstI*-sites in the *epr* gene. Two auxiliary plasmids, *pCBT-epr* and *pCBT(epr::Cm\(^R\))*, were obtained for construction of this linear fragment. The latter plasmid served as a vector for the cloning of the *mpr\(^{B.\text{amy}}\) cassette from pHE52mp, resulting in *pCBT(epr::mpr52)*. The linear *epr::Cm\(^R\) DNA fragment and *pCBT(epr::mpr52)* were used for integration of the second copy of the *mpr\(^{B.\text{amy}}\) cassette and construction of the JE852 (*aprE851, epr::mpr\(^{B.\text{amy}}\)* strain).

The third integration was based on the linear DNA fragment, *nprB::Cm\(^R\)*, carrying the *nprB* gene (the primers-Pr11/Pr12) disrupted by a *HindIII*-generated Cm\(^R\)-carrier amplicon from pC194 (primers-Pr9/Pr10) that was inserted into the unique *HindIII* site in the structural part of *nprB*. Construction of this fragment was provided through formation of the auxiliary plasmid *pCBT(nprB::Cm\(^R\))*). This plasmid was used later as a vector for cloning of the *PstI*-generated *mpr\(^{B.\text{amy}}\) cassette instead of Cm\(^R\) disrupted of *mprB* and construction of *pCBT(nprB::mpr52)*. It was possible so long as the Pr9/Pr10 were designed for bracketing the Cm\(^R\)-marker by (*HindIII-PstI*)(*PstI-HindIII*) sites. The linear DNA fragment, *nprB::Cm\(^R\)*, and the *pCBT(nprB::mpr52)* plasmid were used for construction of the JE852(*aprE851, epr, nprB::mpr\(^{B.\text{amy}}\))* strain that possessed three copies of the *mpr\(^{B.\text{amy}}\)-cassette in the targeted loci of the bacterial chromosome.

### Construction of DNA fragments for random integration of the *mpr\(^{B.\text{amy}}\)-cassette*

A total of 5 \(\mu\)g of chromosomal DNA from *B. subtilis* JE852 was exhaustively hydrolyzed by *BamHI*, followed by self-circularization of the linear DNA fragments by treatment with T4 ligase in 1 mL of reaction mixture. This DNA was then digested by *PstI* and ligated with 5 \(\mu\)g of *PstI*-generated *mpr\(^{B.\text{amy}}\)-Cm\(^R\)*-cassette from pHE52 (*mpr-Cm\(^R\)*) that had been purified from low melting agarose. The ligation mixture was digested by *BamHI*, and about 1 \(\mu\)g of the total DNA was used for the transformation of *B. subtilis* JE852.

### Segregation stability test

About 10\(^7\) cells from overnight cultures of the *B. subtilis* JE852-(*Nxyz::mpr\(^{B.\text{amy}}\)-Cm\(^R\)*) strains were inoculated into 10 mL of fresh LB medium, cultivated for 20 generations and cloned. One hundred individual colonies were tested for Cm resistance. Strains that generated 100% Cm\(^R\) clones after 20 generations were tested for stability after 40 generations and then again after 60 generations. Finally, JE852-(*Nxyz::mpr\(^{B.\text{amy}}\)-Cm\(^R\)*) strains, which generated 100 Cm\(^R\) colonies among the 100 that were tested after 60 generations, were considered to be stable and were used as donors of chromosomal DNA for increasing the *mpr\(^{B.\text{amy}}\)-cassette copy-number.

### Protein analysis

SDS-PAGE was conducted using Laemmli’s method [61] for the evaluation of GSP accumulation in the culture supernatants of *B. subtilis* strains. Gels were stained with Coomassie R-250 and scanned to estimate the protein content with the TotalLab v. 2.01 computer software for determine the portion of GSP among the secreted proteins. Total extracellular protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, USA) in accordance with the manufacturer’s instructions. In addition, the known concentrations of the commercially available carbonic anhydrase from bovine erythrocytes (Sigma) with Mw 29 kDa were used for SDS-PAGE followed by staining and scanning the gel for comparative evaluation of GSP production. Both independent methods gave, practically, coincident results.

### Additional material

#### Additional file 1: Table S1

List of primers used for PCR.

#### Additional file 2: Figure S1

Construction of the linear DNA fragment used for the JE852::aprE::mpr\(^{B.\text{amy}}\) strain construction.

#### Additional file 3: Table S2

Bacterial strains and plasmids used.

### Abbreviations

AntR: antibiotic resistance marker; AprE: alkaline serine protease subtilisin; bp: base pair(s); CCA: carbon catabolite activation; CCC: carbon catabolite control; CCR: carbon catabolite repression; Cm: chloramphenicol; Cm\(^R\): Cm resistance; cr: catabolite responsive element; CSL: corn steep liquor; Em: erythromycin; Em\(^R\): Em resistance; GSP: glutamyl-specific protease, the *mpr* gene product; marker-less strain: a bacterial strain that does not carry an *r* in its genome; *HP*; neutral protease; PCR: polymerase chain reaction; *Pxy*: promoter of the *B. amyloliquefaciens* A-50 *pLU- rpmA* genes; Ter: transcription terminator of the *B. amyloliquefaciens* phaE gene; *mpr\(^{B.\text{amy}}\)* cassette: expression cassette where the structural portion of the *B. amyloliquefaciens* A-50 *mpr* gene is sandwiched between *Pxy* and Ter; SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis; / denotes a plasmid-carrying strain.

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### Authors’ contributions

YAV designed the methods and performed the multi-copy number integrations at random sites of the *B. subtilis* chromosome. EA designed and constructed the recombinant DNA used in this study and drafted the manuscript. LIG tested the level of extracellular GSP accumulation by protein electrophoresis and edited the manuscript. LYG performed the Southern hybridization experiments. SVM coordinated the work and amended the
manuscript. All authors read and approved the final version of the manuscript.

Competing interests
The authors declare that they have no competing interests.

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