**Bacillus SEVA siblings: A Golden Gate-based toolbox to create personalized integrative vectors for Bacillus subtilis**

Jara Radeck, Daniel Meyer, Nina Lautenschläger & Thorsten Mascher

*Bacillus subtilis* combines natural competence for genetic transformation with highly efficient homologous recombination. These features allow using vectors that integrate into the genome via double homologous recombination. So far, their utilization is restricted by the fixed combination of resistance markers and integration loci, as well as species- or strain-specific regions of homology. To overcome these limitations, we developed a toolbox for the creation of personalized *Bacillus* vectors in a standardized manner with a focus on fast and easy adaptation of the sequences specifying the integration loci. We based our vector toolkit on the Standard European Vector Architecture (SEVA) to allow the usage of their vector parts. The *Bacillus* SEVA siblings are assembled via efficient one-pot Golden Gate reactions from four entry parts with the choice of four different enzymes. The toolbox contains seven *Bacillus* resistance markers, two *Escherichia coli* origins of replication, and a free choice of integration loci. Vectors can be customized with a cargo, before or after vector assembly, and could be used in different *B. subtilis* strains and potentially beyond. Our adaptation of the SEVA-standard provides a powerful and standardized toolkit for the convenient creation of personalized *Bacillus* vectors.

The diversity of plasmid vectors in molecular cloning. In molecular cloning, vectors are vehicles to transfer foreign nucleic acids into a living cell. In the context of this article, we restrict the term "vector" to "plasmid vectors", small circular DNA molecules that originate from bacteria. They are easy to handle for inserts up to 10–15 kb, replicate independently of the bacterial chromosome and can be isolated in large amounts through standard plasmid preparation procedures. Over the last decades, vectors were increasingly modified to meet custom needs. For instance, they may contain promoters for ready-to-use gene expression, or reporter genes to measure transcription or translation rates, respectively. More elaborated vector types can contain biosafety features, internal measuring standards or may be used for clean chromosomal gene deletions or dual expression systems. Synthetic biology strives to implement and extend the principles of engineering (standardization, decoupling, abstraction) into biology. Especially standardization in vector and plasmid construction offers the clear advantage of comparability, compatibility, flexibility and reusability of single parts and whole vectors, as exemplified by the Standard European Vector Architecture (SEVA).

The Standard European Vector Architecture provides standardized vectors for Gram-negative bacteria. In 2013, the group of Victor de Lorenzo developed a standardized vector toolbox for the use in Gram-negative bacteria, with a special interest in *Pseudomonas putida*. With SEVA, they set the stage for a community-driven development platform and for evolving a standardized vector collection. This platform facilitates finding, creating, and naming of suitable vectors as well as their downstream handling. Currently, the SEVA database lists 135 SEVA vectors and 49 SEVA siblings (http://seva.cnb.csic.es/, March 2017). Subsequently, linker sequences were developed to make the vectors compatible to different cloning methods.

Each SEVA vector contains at least three functional elements (Fig. 1a), (i) the origin of replication (ori) for vector replication in the host cell, (ii) a selectable marker, e.g. an antibiotic resistance cassette to select for vector...
Figure 1. Configuration of a SEVA vector and its *Bacillus* SEVA sibling. (a) The basic SEVA vector is composed of six parts, separated by defined endonuclease restriction sites. The transcriptional terminators T0 and T1 as well as the origin of transfer oriT are fixed, whereas the cargo, the ori and the antibiotic marker can be chosen freely from a pool of SEVA-compatible parts. (b) For genomic integration, three parts added to the SEVA layout to create the *Bacillus* SEVA sibling pBS: Flanking homology regions up and down, as well as an antibiotic marker for *Bacillus*. Vector verification and propagation occurs in *E. coli* and only the part in between the homology regions (dashed line) will integrate into the genome. Vectors are drawn not to scale. Functional transcriptional units are indicated with an arrow (promoter) and black bar (terminator).

uptake and maintenance, and (iii) a multiple cloning site (MCS) for insertion of the DNA of interest. In the SEVA standard, the latter part is called cargo, irrespective of the size and nature of the insert. The cargo is isolated by flanking double transcriptional terminators (T1, T0), to avoid unwanted transcriptional read-through into other elements of the backbone. All parts are flanked by defined rare endonuclease restriction sites and assemble in a fixed order and orientation (Fig. 1a). They must not contain these and further restriction endonuclease recognition sites. Additionally, the transcriptional terminators and the origin of transfer (oriT, for plasmid conjugation) are predefined, whereas the selectable marker, ori and cargo can be chosen freely, as long as they adhere to the standard. An easy number-based nomenclature assures the fast determination of vector features from the vector’s name.

Distinct features of vectors for *Bacillus subtilis*. *Bacillus subtilis* is the best studied low-G+C Gram-positive bacterium (phylum *Firmicutes*) and one of the leading workhorses of the biotechnological industry. Its ease of genetic manipulation is based on its natural competence, which includes the active uptake of (any) DNA and recombination of homologous regions into the chromosome. These features enable to routinely use integrative instead of replicative vectors. This ensures genetic stability even without maintaining a constant selective pressure. Moreover, copy number effects are avoided, which can otherwise influence the promoter activity on replicative vectors, particularly at the single cell level.

For cloning convenience, vectors designed for integration into the *B. subtilis* genome usually replicate in *E. coli* but do not contain an ori for *B. subtilis*. Instead, two homology regions of at least 400 bp, which define the insertion locus, flank the cargo and the resistance marker for *B. subtilis*. The plasmids are linearized in the *E. coli* part of the vector before *B. subtilis* transformation to avoid integration via single crossing over events. Consequently, only the DNA segment that is located between the homology regions will integrate into the chromosome, resulting in genetic stability of single copy number inserts even in the absence of selective pressure.

The currently available vectors combine known and effective selection cassettes with well-characterized integration loci. This status quo limits the combination of resistance markers and integration sites, and does not allow targeting entirely new chromosomal regions. Moreover, existing vectors cannot be used in another *Bacillus* strain, which differs in the nucleotide sequence at the specified integration site.

Due to the genetic accessibility of *B. subtilis*, PCR-based methods, such as long-flanking homology (LFH)-PCR, can be used to target new or strain-specific loci. While this approach is very convenient for generating knock-out mutants, it is restricted with respect to the cargo: fusing several PCR products, and obtaining large fragments, e.g. the \\textit{luxABCDE} bioluminescence reporter operon (5.7 kb) that allows online measurement, with sufficient yield for efficient transformation (>1 µg) can be tedious. In these cases, transformants have to be sequenced each time to ensure the integrity of the cargo (e.g. lack of mutations). In order to combine the advantages of vectors (stability and reusability) with those of PCR-based methods (flexibility) for the use in *B. subtilis* and related species, we designed, constructed and tested a new vector concept: the *Bacillus* SEVA siblings.

Concept of *Bacillus* SEVA siblings as customized vectors. Here, we describe a toolkit that was developed for the fast and easy generation of genomic integration vectors for *B. subtilis*, thereby overcoming the traditional limitations described above. Each vector contains a resistance marker, cargo and flanking homology regions of choice. To ensure compatibility and reusability of already existing parts for replication in *E. coli*, we
based our system on SEVA and added flanking homology regions as well as Bacillus resistance markers at defined positions (Fig. 1b). Our vectors will be named Bacillus SEVA siblings, which is in line with the current SEVA regulations.

Our toolbox offers seven functional antibiotic resistance cassettes for selection in B. subtilis and high and medium copy number E. coli vectors modified to be assembled with your homology regions of interest in a one-pot Golden Gate assembly. We tested the assembly efficiencies for five different enzymes and the B. subtilis transformability of every part provided. As proof of concept for efficient assembly and functionality, we analyzed the expression levels of the red fluorescing protein mKate2 under control of the xylose-inducible promoter P_{xyl} at different chromosomal insertion loci.

Results

Vector layout for double cross-over homologous recombination in B. subtilis. We designed and constructed our vector building toolkit with the main goal to easily exchange the target loci for double homologous integration into the genome. This is important for two major reasons: Current vector collections do not allow the free combination of integration loci and selectable markers. Moreover, they omit the use of Bacillus strains if they differ from the reference lab strains in their nucleotide sequence, so that the integration sites of standard vectors are not compatible. For our collection of customizable vectors, we focus on allelic replacement via double recombination, where the DNA-sequence to be integrated into the chromosome (here termed integration part) is flanked by two regions of homology (Fig. 1b). For B. subtilis, approximately 400 bp of identical sequence are required for efficient integration. Shorter sequences (70 bp) can sometimes be sufficient, but are not recommended due to the significantly reduced efficiency. For ease of construction, the integration part is combined with an ori and selectable marker for E. coli (here termed replication part). Before B. subtilis transformation, the vector is linearized in the replication part to avoid single cross-over events and ensure double cross-over integration.

Although our toolkit was optimized for the exchange of integration sites, it can be customized in manifold ways as will be outlined in the discussion. A quick user manual with helpful information for vector construction can be found in the supplemental material (Text S1).

The customizable vector collection is based on SEVA to allow re-use of E. coli parts. Currently, most vectors and plasmids used for genetic manipulation of B. subtilis are propagated in E. coli. As demonstrated for NarK and β-carotene production, the E. coli vector propagation strongly depends on the ori and resistance markers in ways that cannot be foreseen yet. Consequently, we designed our vectors to be flexible not only with respect to their cargo, but also their ori and E. coli resistance marker. For this purpose, we based our vector toolkit on SEVA. This standard was designed for vectors to allow exchanging of the cargo, resistance marker and ori, using defined rare type II restriction enzymes. The accompanying vector collection was very well-received by scientists and is still growing. It can be used to adapt the vectors of our toolbox, which are therefore named Bacillus SEVA siblings. SEVA restriction sites were removed from critical parts of the entry vectors, so that the final vectors adhere to the SEVA standard, if no forbidden restriction sites are present in the customized parts.

Bacillus SEVA siblings are assembled de novo from multiple fragments via Golden Gate cloning. As depicted in Fig. 1b, the integration and replication parts of an integration vector are separated by the regions of homology, called up and down. Consequently, the exchange of both up and down fragments with standard cloning techniques would involve two cloning and verification steps without a selectable marker. To avoid laborious stepwise cloning, advanced cloning techniques for the easy, fast, efficient and directed one-pot assembly of multiple fragments are available, such as Gibson assembly or Golden Gate cloning. The latter is based on a ligase and type IIS endonucleases (e.g. BsaI), which cut outside (next to) their recognition site. Consequently, the restriction site can be designed according to need and separated from the recognition site during the cloning procedure. The reaction mix can include linear and circular DNA, containing restriction sites for the same enzyme but different overhangs. This allows the easy and efficient assembly of up to ten parts in the correct order and loss of the recognition sites in the final vector. For our vector toolbox, we chose Golden Gate assembly for the following reasons: (i) Gibson assembly usually must be established in a lab to run smoothly, whereas restriction based cloning is more robust. (ii) The up and down regions need to be PCR-generated and Gibson assembly asks for longer overhangs thus increasing the primer costs.

Instead of re-using and modifying preexisting vectors, each new vector will be freshly assembled by combining four fragments via Golden Gate assembly (Fig. 2): the up and down fragments, the replication part, and the cargo which includes an antibiotic marker for selection in Bacillus and a MCS. Therefore, all vectors and sequences offered through the Bacillus Genetic Stock Center (BGSC) and the SEVA collection (see Table 1 and supplementary data S2) are entry vectors to allow customized assembly, but no final Bacillus vectors.

Architecture of Bacillus SEVA siblings. Figure 1 compares a final Bacillus SEVA sibling (pBS), with a standard SEVA vector for E. coli. SEVA suggests a designated location for the insertion of special features that are not part of the cargo. They are positioned at the terminator sequences and next to, but not obstructing the AscI and SwaI recognition sites which allow the exchange of SEVA vector parts. In line with this regulation, the homology regions and resistance marker are located at both terminator sequences. The respective Bacillus resistance cassette is placed between the cargo and the T0-terminator and directed counter-clockwise in order to not interfere with the transcription of the cargo. SEVA-vectors are named according to their features in a number-based code, see SEVA 2.0 for a comprehensive description. We suggest the naming of final vectors to be based on the SEVA-standard, in which the E. coli features are specified in three digits: the first digit describes the E. coli resistance marker, e.g. 1 for ampicillin resistance or 2 for kanamycin. The second digit indicates the ori, e.g.
Golden Gate shuffling to assemble Bacillus SEVA siblings with type IIS restriction enzymes. In our current set-up, four different fragments are needed to assemble the final vector: the up and down homology fragments, the cargo with the Bacillus resistance cassette and the part for replication and selection in E. coli, which we call destination vector. These entry parts can be combined using Golden Gate assembly as detailed in Fig. 2. The up and down homology fragments can be used either as PCR products (as depicted) or as cargo of the specialized vectors containing the up (pSEVA243X) and down (pSEVA243Y) fragments, respectively. Each entry part is flanked by recognition sites for a type IIS restriction endonuclease, which creates overhangs that allow for the directional assembly of all entry parts. Compatible overhangs are named with the same capital letter in Fig. 2a.

As necessary for Golden Gate assembly, the recognition sites are located “outside” of the part desired for the assembly, so that correctly assembled parts cannot be re-cleaved – in contrast to the re-ligation products of entry vectors. By this means, assembly of mostly correct final vectors is ensured. The destination vector carries a different antibiotic marker than all other entry vectors to select for vectors carrying the correct backbone. An mRFP1-cassette present on the original destination vector is used for red/white screening. This part is removed during assembly of the final vector so that colonies carrying the original vector appear red and colonies carrying the correct final vector appear white.

Classic Golden Gate assembly uses BsaI and BbsI (=Bpil) as type IIS restriction enzymes24,28, but BsmBI and BtgZI were recently found to also be suitable29,30. Our Bacillus SEVA siblings toolbox accommodates all four of them for assembly to circumvent compatibility issues with the desired genomic region, e.g. the presence of one or more recognition sites in the up and down fragments. In addition to BsaI, BbsI, BsmBI and BtgZI, we also included a fifth restriction enzyme (AarI) – not reported previously for its use in Golden Gate assembly.

Golden Gate assembly restriction sites are arranged in special MCS-IIS. All four entry parts are flanked by MCSs that contain recognition sites for all five type IIS restriction enzymes (MCS-IIS). Inside each MCS-IIS, recognition and restriction sites are designed so that the same overhang sequence is created, independent of the enzyme used. The overhangs are non-palindromic and differ in at least two nucleotides to ensure the correct assembly of the desired vector. For ease of understanding, fusion sites were named with capital letters B, C, E, and F as indicated in Fig. 2. Figure 3 shows the annotated MCS-IIS C2, in which all five enzymes create the overhang GCGA. For the detailed sequence of all 8 MCS-IIS, see Fig. S1.

To compare the assembly efficiency as a function of the enzyme of choice, we used one set of entry vectors to construct final vectors with identical features. However, nucleotide sequences differ at the assembly scar sites, due to the MCS-IIS. We used pSEVA23X-amyE (up), pSEVA243Y-amyE (down), pBSc243M and pBSd141R as entry vectors for pRO1600/ColE1 or 9 for pBR322/ROP. The third digit encodes the cargo, e.g. 1 for the default MCS or 3 for the lacZα-pUC18 MCS. pSEVA243 consequently is a vector mediating kanamycin resistance [2] with a high copy number [4 = pRO1600/ColE1] that carries a MCS for blue/white screening [3 = lacZα-pUC18]. Bacillus-specific features should be added behind, so pBS143K-amyE is a vector with an E. coli ampicillin resistance marker [1], pRO1600/ColE1 ori [4] and lacZα-pUC18 MCS [3] that carries a kanamycin Bacillus resistance marker [K] and integrates into the amyE-locus [amyE].

We conceptualized the assembly so that SEVA-parts can be exchanged before or after the assembly of the final vectors, e.g. to accommodate the presence of either SEVA- or Golden Gate-forbidden restriction sites.
### Vectors for default assembly

#### Destination vectors

| Name          | Description                                                                 | Resistance in E. coli / B. subtilis | Source |
|---------------|-----------------------------------------------------------------------------|-------------------------------------|--------|
| ECE701 pBSd141R | mRFP1, MCS-IIS F2, bla, ori pRO1600/ColE1, MCS-IIS B1                        | Amp′/—                             | This study |
| ECE702 pBSd191R | mRFP1, MCS-IIS F2, bla, ori pBR322/ROP, MCS-IIS B1                           | Amp′/—                             | This study |

#### Vectors for flanking homology regions

| Name          | Description                                                                 | Resistance in E. coli / B. subtilis | Source |
|---------------|-----------------------------------------------------------------------------|-------------------------------------|--------|
| ECE703 pSEVA243 | lacZ+ pUC18 MCS, neo, ori pRO1600/ColE1                                       | Kan′/—                             | SEVA² |
| ECE704 pSEVA243X | lacZ+ + pUC18 MCS incl. MCS-IIS B2 + C1 for up, neo, ori pRO1600/ColE1       | Kan′/—                             | This study |

### Cargo-Resistance vectors

#### Destination vectors

| Name          | Description                                                                 | Resistance in E. coli / B. subtilis | Source |
|---------------|-----------------------------------------------------------------------------|-------------------------------------|--------|
| ECE706 pBSc241B | MCS-default, MCS-IIS E1, neo, ori pRO1600/ColE1, MCS-IIS C2, bleO            | Kan′/ble′                          | This study |
| ECE707 pBSc241C | MCS-default, MCS-IIS E1, neo, ori pRO1600/ColE1, MCS-IIS C2, cat             | Kan′/cm′                           | This study |
| ECE708 pBSc241M | MCS-default, MCS-IIS E1, neo, ori pRO1600/ColE1, MCS-IIS C2, ermC            | Kan′/MLS′                          | This study |
| ECE709 pBSc241S | MCS-default, MCS-IIS E1, neo, ori pRO1600/ColE1, MCS-IIS C2, aad(9)          | Kan′/spc′                          | This study |
| ECE710 pBSc241T | MCS-default, MCS-IIS E1, neo, ori pRO1600/ColE1, MCS-IIS C2, tetL            | Kan′/tet′                          | This study |
| ECE711 pBSc241Z | MCS-default, MCS-IIS E1, neo, ori pRO1600/ColE1, MCS-IIS C2, ble-Sh         | Kan′/zeo′                          | This study |
| ECE720 pBSc291K | MCS-default, MCS-IIS E1, neo, ori pBR322/ROP, MCS-IIS C2, Aph(3′) IIIa        | Kan′/kan′                          | This study |
| ECE721 pBSc243B | lacZ+ + pUC18 MCS, MCS-IIS E1, neo, ori pRO1600/ColE1, MCS-IIS C2, bleO      | Kan′/ble′                          | This study |
| ECE722 pBSc243C | lacZ+ + pUC18 MCS, MCS-IIS E1, neo, ori pRO1600/ColE1, MCS-IIS C2, cat       | Kan′/cm′                           | This study |
| ECE723 pBSc243M | lacZ+ + pUC18 MCS, MCS-IIS E1, neo, ori pRO1600/ColE1, MCS-IIS C2, ermC       | Kan′/MLS′                          | This study |
| ECE716 pBSc243S | lacZ+ + pUC18 MCS, MCS-IIS E1, neo, ori pRO1600/ColE1, MCS-IIS C2, aad(9)   | Kan′/spc′                          | This study |
| ECE717 pBSc243T | lacZ+ + pUC18 MCS, MCS-IIS E1, neo, ori pRO1600/ColE1, MCS-IIS C2, tetL      | Kan′/tet′                          | This study |
| ECE718 pBSc243Z | lacZ+ + pUC18 MCS, MCS-IIS E1, neo, ori pRO1600/ColE1, MCS-IIS C2, ble-Sh    | Kan′/zeo′                          | This study |
| ECE721 pBSc293K | lacZ+ + pUC18 MCS, MCS-IIS E1, neo, ori pBR322/ROP, MCS-IIS C2, Aph(3′) IIIa | Kan′/kan′                          | This study |

### Vectors for customizable resistance markers

| Name          | Description                                                                 | Resistance in E. coli / B. subtilis | Source |
|---------------|-----------------------------------------------------------------------------|-------------------------------------|--------|
| ECE705 pBSc241 | MCS-default, MCS-IIS E1, neo, ori pRO1600/ColE1, MCS-IIS C2                   | Kan′/—                              | This study |
| ECE712 pBSc243 | lacZ+ + pUC18 MCS, MCS-IIS E1, neo, ori pRO1600/ColE1, MCS-IIS C2             | Kan′/—                              | This study |
| ECE719 pBSc291 | MCS-default, MCS-IIS E1, neo, ori pBR322/ROP, MCS-IIS C2                      | Kan′/—                              | This study |
| ECE725 pBSc293 | lacZ+ + pUC18 MCS, MCS-IIS E1, neo, ori pBR322/ROP, MCS-IIS C2                | Kan′/—                              | This study |
| ECE722 pBSc391 | MCS-default, MCS-IIS E1, cat, ori pBR322/ROP, MCS-IIS C2                       | Cm′/—                               | This study |
| ECE726 pBSc393 | lacZ+ + pUC18 MCS, MCS-IIS E1, cat, ori pBR322/ROP, MCS-IIS C2                | Cm′/—                               | This study |

### Plasmids for activity-test

| Name          | Description                                                                 | Resistance in E. coli / B. subtilis | Source |
|---------------|-----------------------------------------------------------------------------|-------------------------------------|--------|
| pSEVA243X-amyE | pSEVA243X-derivative carrying a 550 bp amyE ap fragment                      | Kan′/—                              | This study |
| pSEVA243Y-amyE | pSEVA243Y-derivative carrying a 580 bp amyE down fragment                    | Kan′/—                              | This study |
| pBSc241M_P_up, mKate2 (2195) | pBSc241M-derivative carrying mKate2 under the control of xylose-inducible P_up        | Kan′/—                              | This study |
| pBS141M-amyE_mKate2 | P_up mKate2, amyE, bla, ori pRO1600/ColE1, amyE, erm                      | Amp′/MLS′                           | This study |
| pBS141M-ypqP_mKate2 | P_up mKate2, ypqP, bla, ori pRO1600/ColE1, ypqP, erm                     | Amp′/MLS′                           | This study |
| pBS141M-ykoS_mKate2 | P_up mKate2, ykoS, bla, ori pRO1600/ColE1, ykoS, erm                   | Amp′/MLS′                           | This study |

Continued
Table 1. Vectors of the Bacillus SEVA siblings toolbox. *Bacillus* Genetic Stock Center (BGSC, http://www.bgsc.org/). The vector names act as identifiers for the SEVA or SEVA siblings collection. **Plasmid. **Bacillus SEVA sibling, **d** destination vector, **c** cargo vector, **f** final vector. Numbers according to SEVA standard: 1st position, resistance marker (1, amp. 2, kan). 2nd position, origin of replication (4, pRO1600/ColE1, a narrow-host-range ori with high copy number in *E. coli* and varying copy number in *Pseudomonas aeruginosa* and close relatives). 9, pBR322/ROP (medium copy number ori in *E. coli* and few other bacteria). 3rd position, cargo (1, MCS default. 3, lacZ-pUC18 MCS which allows for blue-white screening with X-Gal). *lacZa*, premature stop codon (C02A, Q68Stop). lacZa**, premature stop codon (pSEVA243X: G390A, W130Stop, pSEVA243Y: G399A, W133Stop). Both variants are still suitable for blue-white screening. Genes encoding antibiotic resistance markers are explained in detail in Table 3. #Flanking homology regions can be stored in those vectors. If the PCR fragment contains the restriction sites needed for assembly, it can be ligated blunt end into pSEVA243 via Smal. If restriction sites should be added for all enzymes, the PCR fragments can be ligated blunt end into those vectors. In this case, the cargo carries a lacZa-fragment that allows for blue-white screening and colonies carrying the correct vector appear blue in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Red colonies carry the original destination vector and white colonies an incorrect vector. We checked colony color, test digest, sequencing and transformation results are given in total. **Test digest, sequencing and transformation results are given in total. **Flanking homology regions can be stored in those vectors. If the PCR fragment contains the restriction sites needed for assembly, it can be ligated blunt end into pSEVA243X (for up) or pSEVA243Y (for down) via EcoRV. In this case, the correct orientation needs to be verified by sequencing.

Table 2. Assembly efficiencies depending on restriction enzyme. *Entry parts: pBSc243M, pBSd141R, pSEVA243X-amyE (up) and pSEVA243Y-amyE (down). **Colonies appear blue if carrying the correct final vector and red if the destination vector is unchanged. If available, only blue colonies were used for test digest and correct test digests were used for sequencing. Data is shown for 3 independent Golden Gate assemblies with AarI, BbsI, Bsal or BsmBI. The total number of colonies as well as their apparent color is shown as average and standard deviation. BtgZI did not lead to correct assemblies of final vector and data is derived from a single experiment only. **Test digests, sequencing and transformation results are given in total. **Flanking homology regions can be stored in those vectors. If the PCR fragment contains the restriction sites needed for assembly, it can be ligated blunt end into pSEVA243X (for up) or pSEVA243Y (for down) via EcoRV. In this case, the correct orientation needs to be verified by sequencing.

### Table 1: Vectors used for vector construction (templates for PCR reactions)

| Enzyme | % Blue | % White | % Red | Total colonies | Corr. test digest | Corr. Sequencing | B. subtilis transf. |
|--------|--------|---------|-------|----------------|------------------|-----------------|-------------------|
| AarI   | 36.8 ± 26.1 | 0.9 ± 0.9 | 62.2 ± 25.5 | 3017 ± 1292 | 14/18 | 3/5 | ++/− | 4/4 |
| BbsI   | 41.5 ± 34.2 | 0.7 ± 0.1 | 57.7 ± 34.1 | 3568 ± 1084 | 15/18 | 3/5 | ++/− | 4/4 |
| Bsal   | 52.8 ± 13.1 | 6.1 ± 2.2 | 41.1 ± 13.9 | 4004 ± 1443 | 15/18 | 3/5 | ++/− | 4/4 |
| BsmBI  | 49.8 ± 11.0 | 3.7 ± 0.5 | 64.5 ± 10.9 | 2000 ± 412 | 13/18 | 3/3 | ++/− | 4/4 |
| BtgZI  | 0 ± 0 | 100 | 0 | 182 | 0/6 | 0/3 | n.a. | n.a. |
Our collection of entry parts for the assembly of Bacillus SEVA siblings. For the assembly of Bacillus SEVA siblings vectors, four different categories of entry parts are needed: cargo and resistance, up, down, and the replication part. They will be described in more detail below. The entry vectors offered with our toolbox are depicted in Fig. 4 and listed with detailed descriptions in Table 1.

**pBSc: Cargo and resistance.** Since for most experiments the same cargo will usually be combined with the same resistance marker, both are located on the cargo vector pBSc. As a result, only four instead of five parts have to be assembled, thereby increasing cloning efficiency. For our collection of cargo vectors, we used well-established antibiotic resistance markers to enable selection on phleomycin D, chloramphenicol, kanamycin, macrolide and streptogramin B antibiotics (MLS), spectinomycin, tetracycline, and zeocin (Table 3). Forbidden restriction sites were removed and transcriptional terminators were added where necessary. Transcription occurs in the opposite direction to the cargo. Per default, the cargo vector uses the high copy number ori pRO1600/ColE1. For the kanamycin resistance marker, this vector was unstable, so the medium copy number ori pBR322/ROP was used. All cargo vectors are offered with the default SEVA MCS (pUC18-related polylinker without lacZα) or the lacZα*-pUC18 MCS (Table 1). The cargo of choice can be inserted into the cargo vector or into the final vector, depending on enzyme compatibility and cloning strategy.

| Abbr. | Gene | Description | Antibiotic | Conc. [µg ml⁻¹] | Source |
|-------|------|-------------|------------|----------------|--------|
| | amp’ | bla | β-lactamase (Eco) | Ampicillin | 100 | pSEVA1437 |
| | kan’ | neo | neomycin-kanamycin phosphotransferase type I (Eco) | Kanamycin | 50 | pSEVA2417 |
| cm’ | cat | chloramphenicol O-acetyltransferase (Eco) | Chloramphenicol | 35 | pSEVA3417 |
| | B | ble’ | bleO’ | bleomycin binding protein (phleomycin D) | Phleomycin D1 | 100 | pDG14848 |
| | C | cm’ | cat | chloramphenicol O-acetyltransferase | Chloramphenicol | 5 | pBS3Clux17,19 |
| | K | kan’ | aph(3’)-IIla | aminoglycoside O-phosphotransferase APH(3’)-IIla | Kanamycin | 10 | pDG78048 |
| | M | MLS’ | ermC | 23 S rRNA (adenine(2058)-N(6))-methyltransferase | Erythromycin & Lincomycin | 1 & 25 | pDG64745 |
| | S | spc’ | aad(9) | aminoglycoside nucleotidyltransferase ANT9 | Spectinomycin | 200 | pBS4585,67 |
| | T | tet’ | tetL | tetracycline efflux MFS transporter | Tetracycline | 12.5 | pDG151345 |
| | Z | zeo’ | ble-Sh’ | phleomycin/bleomycin binding protein (codon-optimized for Bsu) | Zeocin | 100 | This study |

**Table 3.** Description of antibiotic markers used in this study. *Eco, E. coli; Bsu, B. subtilis. 55 µg ml⁻¹ were used for medium copy number vectors. *bleO and ble-Sh both mediate resistance against phleomycin or zeocin (both from the bleomycin family) by binding to the antibiotic, respectively. There are differences in amino acid sequence of the encoded proteins and in the properties of the antibiotics, but mediation of cross-resistance cannot be excluded.
up and down flanking homology regions. The choice of up and down flanking homology regions depends on and needs to be adjusted to the Bacillus strain and experiment. The regions need specific overhangs for the subsequent assembly. This can be achieved by either of the following two strategies: (i) PCR-amplified fragments of choice (without overhangs) are cloned blunt end into the EcoRV-linearized pSEVA243X (up) or pSEVA243Y (down), respectively. As a result, the insert receives the matching MCS-IIS encoded on these vectors which can be used for Golden Gate assembly. (ii) Incorporation of one restriction site to the primers will directly allow the use of the PCR product for Golden Gate assembly.

pBSd: replication part in E. coli. The destination vector carries the replication part of the final vector. Its resistance (ampicillin marker) differs from all other entry vectors to allow selection for the correct backbone after vector assembly. A high copy number (pBSd141R) and medium copy number version (pBSd191R) are provided. Both carry an mRFP cassette in their default MCS, thereby allowing red/white screening. After Golden Gate assembly, colonies with the original destination vector will appear red and therefore can be discarded. If different E. coli features are required for the final vector, they can be exchanged in the destination vector or the final vector, depending on needs and enzyme compatibilities.

Assembly, transformation and integration of pBS vectors. The reaction for the vector assembly contains the entry parts, one of the type IIS restriction enzymes, ligase and buffer. The specific protocols depend on the enzyme used and can be found in the Materials and Method section. Competent E. coli cells are subsequently transformed with the reaction mix and plated on selective media containing ampicillin (and X-Gal in case of blue/white screening). Verification of the final vector can be achieved in a two-step procedure using test digests followed by sequencing. For the latter, primers TM3782 and TM3783 (if pBSd141R was used as destination vector) or TM3783 and TM5128 (pBSd191R) are recommended. The sequencing results should cover the up and down region as well as the adjacent assembly scars to ensure correct assembly of all parts. We tested all entry parts of the toolbox (Table 1) for their assembly efficiencies (Tables S4–S6) and found that in all cases it was more than sufficient to test six colonies of the correct color to obtain a correct final vector. If the high copy number destination vector is used, or selection for the correct cargo is possible, e.g. via blue/white screening, assembly efficiencies are even higher.

Prior to transformation of B. subtilis W168, all vectors have to be linearized, e.g. using AplI, to ensure chromosomal integration by double homologous recombination. All transformations were successful – except for vectors carrying the tetracycline resistance marker where only for one of two loci could be targeted (Tables S4, S5). The transformation efficiencies depended on the selection marker (Tables 2, 3 and S4–S6). Correct integration was verified by colony PCR or physiological tests: e.g. a starch test for integration into the amyE locus.

As a proof of concept, we analyzed the expression of the reporter gene mKate2 under control of the xylose-inducible promoter Pxylose at five loci spread across the chromosome (Table 4): amyE, ykoS, ypqP (prophage SP3), ndk, and thrC at positions 28, 111, 183–195, 203, and 283° on the circular chromosome, respectively. amyE and thrC are early-discovered and frequently used integration loci close to the ori, encoding a starch-hydrolyzing alpha-amylase and the threonine synthase, respectively. The 130 kb-large prophage SP3, which itself is inserted at the ypqP locus, is known to be not essential and was targeted to demonstrate the possibility of deleting large genomic regions. The two remaining genes, ndk and ykoS, encode a nucleoside diphosphate kinase and a gene of unknown function, respectively. Those non-essential genes were chosen based on their chromosomal location. Since the same reporter was used in all vector constructs, the reporter-cargo was added at the entry vector level to pBSc241M, resulting in pBSc241M-Pxylose-mKate2. pBSd141R (ori pR0/ColEl, high copy number) was chosen as the destination vector and primers were designed for PCR-product assembly of up and down fragments via BsaI. Assembly and verification of four final vectors was achieved within five working days. The vector carrying homology regions for integration into ndk was instable (small colonies), necessitating a change to pBSd191R (ori pBR322/ROP, medium copy number) as a destination vector. After transformation of B. subtilis W168, the resulting integrants were verified by starch or threonine auxotrophy tests (amyE and thrC, respectively), or colony PCR for the remaining loci (Table 4). The xylose-inducible promoter Pxylose was fully induced with xylose in exponentially growing cultures. The fluorescence intensity of B. subtilis cells was quantified in triplicates in a microtiter plate reader as a measure for mKate2 production (Fig. 5). The results highlight a dependence of the expression levels on the chromosomal location: As demonstrated previously, genes close to the ori (0°/360°) tend to be expressed at higher levels than those close to the termination region (180°). One exception was the ykoS-locus, located at the replication termination site, which had a higher reporter activity than the constructs inserted at the ori-proximal ypqP or ndk sites. Nevertheless, these results are in good agreement with a recent study31, thereby demonstrating that our vector toolbox can for example be used to study the effect of chromosome location on expression levels in a simple and straightforward manner.

Discussion
B. subtilis is a versatile heterologous host with powerful genetics allowing precise genomic manipulation. But so far, the available integrative vectors could not easily be adapted to different strains or, more importantly, other bacilli, since no modular toolbox was available to allow free combination of resistance markers and integration sites. To fill this gap, we present a rigorously evaluated toolbox for the construction of integrative vectors with customizable flanking homology regions for Bacillus sp. The final pBS vector can be assembled within one week from four entry parts via efficient Golden Gate cloning. Our vector suite offers the choice of four type IIS restriction endonucleases (AarI, BbsI, BsaI and BsmBI) for Golden Gate assembly, seven Bacillus antibiotic resistance markers, and two different MCSs, available on either a high or medium copy number E. coli backbone. The toolbox is adjusted to and widely compatible with the E. coli SEVA standard to allow reusability of its cargos or vector
Figure 4. Vector suite for the generation of Bacillus SEV A siblings. Schematic representation of the vector architectures, details are listed in Table 1. (a) Vectors for flanking homology regions. Up fragments (PCR product) can be stored in pSEV A243X and down in pSEV A243Y, each linearized with EcoRV. The respective MCS-IIS are encoded on the vectors. If required restriction site are already encoded on the primer overhangs, fragments can be stored in pSEV A243 or used directly for Golden Gate assembly. (b) Cargo vectors carry one of the following Bacillus antibiotic markers: Ble, Cat, Kan, MLS, Spc, Tet, Zeo and either the default MCS (pBSc241res) or the lacZα-pUC18 MCS for blue/white-screening. Vectors carrying the Bacillus kanamycin resistance marker utilize the medium copy number pBR322/ROP ori, all others the high copy number pRO1600/ColE1. Backbones are also available without Bacillus marker to allow insertion of a new or customized marker. (c) Destination vectors carry an mRFP1-cassette as cargo for red/white screening and an ampicillin resistance marker for selection in E. coli. They are available with high (pRO1600/ColE1) or medium (pBR322/ROP) copy number origins of replication.
parts. All components provided in this toolbox were successfully tested for assembly efficiency, functionality, and usability in *B. subtilis*. The supplementary assembly guide (Text S1) provides necessary information to facilitate a fast and efficient cloning process.

In the course of developing our vectors, a few combinations were discovered which were difficult to handle on high copy number vectors (ori pRO1600/ColE1): (i) The *Bacillus* kanamycin resistance marker was prone to mutations and is therefore offered with the medium copy number ori pBR322/ROP. (ii) The *lacZα*-pUC18 MCS differs from the original *lacZα*-pUC18 MCS in a single-nucleotide polymorphism causing a premature stop codon in *lacZα* that does not impair blue/white screening. There was a strong selection pressure against the original *lacZα*-pUC18 MCS (very small colonies), which caused transposon integrations and single nucleotide polymorphisms in *lacZα*. Consequently, we used the mutated but fully functional *lacZα*-pUC18 MCS for our constructs. (iii) The final ndk-vectors only contained the ndk down fragment when using the high copy number pBSd141R as destination vector. However, assembly with the medium copy number vector pBSd191R was successful in the first attempt.

Based on our experience, the use of a medium copy number vector is highly recommended as the first troubleshooting strategy in case of cloning issues, especially if slowly growing colonies occur. Also, low copy number oris (e.g. p15A and pSC101) or different directionality of the inserts could be used in case toxicity issues occur, which result in genetic instabilities even on medium copy number vectors.

Furthermore, the tetracycline resistance marker could not confer resistance in *B. subtilis* W168 when the amyE locus was targeted. However, when using a different integration locus (*lacA*), the transformation was successful. Due to the dependence of expression levels on the chromosomal region, we suspect this to be causing the transformation issues.

| Locus   | % Light red  | % White | % Red | Total colonies | Correct test digest | Correct Sequencing | *B. subtilis* trans. |
|---------|--------------|---------|-------|----------------|--------------------|--------------------|--------------------|
| amyE    | 69.8         | 19.8    | 10.3  | 1160           | 6/6                | 1/1                | +                  |
| ypsP    | 92.2         | 2.9     | 4.9   | 1236           | 6/6                | 1/1                | + / 4/4            |
| ykaS    | 91.6         | 3.2     | 5.3   | 1900           | 6/6                | 1/1                | + / 4/4            |
| ndk     | 75           | 25      | 25    | 496            | 12/12              | 1/1                | +                  |
| thrC    | 92.1         | 5.2     | 2.6   | 4580           | 6/6                | 1/1                | + / 4/4            |

Table 4. Assembly efficiencies* and reporter activity depending on integration sites. *Data is shown for one Golden Gate assembly using BsaI and the following entry parts: PCR products up and down with BsaI-overhangs, pBSd141R and pBSc141M_pXyl-mKate2. *Colonies appear light red if carrying the correct final vector and red if the destination vector is unchanged. Only light red colonies were used for test digest and correct test digests were used for sequencing. The correct chromosomal integration was verified as described in Material and Methods. *Number of colonies per 100 µl of transformation mixture: +++, >1000; +, >100 and number of colonies with verified chromosomal integration from number of tested colonies. *pBSd191R was used as destination vector. No colonies of “light red” color could be identified due to lower copy number of the final vector, in comparison to the other constructs. As a consequence, white colonies were used for further verification.

Figure 5. Maximal promoter activity of P<sub>xylA</sub>-mkate2 depends on chromosomal location. The reporter construct P<sub>xylA</sub>-mkate2 was integrated into the *B. subtilis* genome at five different chromosomal loci, as indicated. mKate expression was maximally induced with the addition of 0.2% xylose and fluorescence intensity was measured as an indicator for mKate abundance. The fluorescence intensity is given as a function of the chromosome position. The error bars show the standard deviation of three independent biological replicates. The data was fitted to two second order polynomial functions: dashed line: no constrains, R² = 0.85; dotted line: minimum was set to X = 180, R² = 0.72.
The Bacillus SEV A siblings toolbox was developed to provide a versatile starting point for the efficient construction of personalized, yet standardized vectors. Both, the entry parts as well as final vectors can be customized to meet personal needs. The MCS or cargo and ori can easily be exchanged according to the SEV A standard and new or modified resistance markers can be inserted in markerless cargo vectors via AscI and MluI, e.g. markers flanked with target sites for recombinases. These systems would allow directed, recombinase-mediated removal of the marker after chromosomal integration (for reviews on recombinase-mediated cassette exchange, e.g. by Flp and Cre/loxP, see[32,33]).

It should also be pointed out that the free choice of chromosomal integration loci provided by our vector toolbox allows for replacing even larger (non-essential) chromosomal areas directly in the process of plasmid integration. We have demonstrated this possibility by integrating an mkate2-expressing plasmid into the ypqP locus of the B. subtilis chromosome, thereby deleting the 130 kb prophage SP (Table 4). This combined integration/deletion at any desired chromosomal position will greatly enhance the possibilities in genetically manipulating B. subtilis.

The current resistance markers located on cargo vectors were tested for their functionality only in B. subtilis W168. Since they originate from broad host range vectors, they are expected to be functionally expressed in many low G + C Gram-positive bacteria (phylum Firmicutes). The Bacillus SEV A siblings might therefore be suitable for e.g. other bacilli, increasing the number of species where customized vectors can be used for genetic manipulation. Indeed, preliminary results from an ongoing study using our vectors reported similar or even better vector assembly efficiencies as those described in Table 2. Choromaphenicol- or erythromycin-resistant mutants were readily obtained in Paenibacillus polymyxa (order: Bacillales, family: Paenibacillaceae) (Christoph Engl, personal communication).

If transformation efficiencies are too low, conjugation can be performed using the oriT already included in the SEV A standard, which provides efficient transfer into various Gram-negative and -positive hosts[34,35]. It can be exchanged with a conjugation marker of choice in the destination or final vector to meet the needs of the target organism. Recombination efficiency of homologous sequences into the chromosome varies more than 30-fold along the B. subtilis chromosome[36], and even more between species. In general, integration rates can be improved by using longer stretches of DNA or a vector with a temperature-sensitive replicon (e.g. based on pMAD[37]). Vector replication not only increases the number of vectors per cell and number of cells carrying a vector (by passing it on to the next generation), but also supports the second recombination event during which the vector backbone is excised from the chromosome[38]. A temperature-sensitive origin of replication can therefore be added to the destination or final vector to improve recombination with the chromosome.

Moreover, the construction logic described for the Bacillus SEV A siblings – that is, the restriction enzymes used for the assembly of the final integrative plasmids – can of course also be applied for developing other SEV A-compatible integration vectors for completely unrelated microorganisms in a similar fashion. But this would require adjusting the corresponding resistance cassettes applicable to these bacteria.

Here, we present the first fully modular, yet standardized vector toolkit for integrative vectors in B. subtilis and beyond. We hope that the Bacillus SEV A siblings vector toolbox will prove to be useful for projects throughout the Bacillus world. In case personalized entry vectors (pBSc, pBSd) are created, we would like to encourage sharing them with the Bacillus community, e.g. via the SEV A or BGSC collections, to further improve the tools available for genetically manipulating these powerful organisms.

Material and Methods

Bacterial strains and growth conditions. All strains used in this study are listed in Table S1. B. subtilis and E. coli were routinely grown in lysogeny broth (LB) medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) at 37 °C with agitation (220 rpm). Solid media additionally contained 1.5% (w/v) agar. Selective media contained appropriate antibiotics, as provided in Table 3.

Transformation. E. coli (XL1 blue, Agilent Technologies, Santa Clara, CA, USA) competent cells were prepared and transformed according to the rubidium chloride method[39,40], achieving ~5*10⁶ colony forming units (CFU) per µg pUC18 DNA. Transformations of B. subtilis were carried out as described previously[41,42]. The integration of plasmids into the B. subtilis genome was checked on starch plates (amyE), with minimal medium lacking threonine (thrC) or colony PCR (lacA, ypqP, ykoS, ndk). Detailed protocols were published previously[43,44].

DNA manipulation. Vectors and plasmids used in this study are listed in Table 1. General cloning procedure, such as endonuclease restriction digest, ligation and PCR, was performed with enzymes and buffers from New England Biolabs® (NEB; Ipswich, MA, USA) or Thermo Scientific™ (Waltham, MA, USA) according to the respective protocols. Phusion® polymerase was used for PCRs if the resulting fragment was further used, otherwise OneTaq® was the polymerase of choice. PCR-purification was performed with the HiYield PCR Gel Extraction/PCR Clean-up Kit (Süd-Laborbedarf GmbH (SLG), Gauting, Germany). Plasmids were prepared using alkaline lysis and subsequent DNA precipitation. All plasmids created during this study are listed in Table 1, their construction is described in supplemental Table S2 and all primer sequences are given in Table S3.

Golden Gate assemblies of final vectors. Golden Gate assemblies were performed using T4 DNA ligase (30 WU) from Thermo Scientific™ with the accompanied buffer. BsaI, BbsI, BsmBI and BtgZI were purchased from NEB, AarI from Thermo Scientific™. Entry parts were diluted to 20 or 40 nM stock concentrations and 2 or 1 µl were used per 15 µl reaction, respectively. For all enzymes, 0.5 µl were used per reaction, except for AarI where 1.5 µl were necessary because of its lower activity. BsaI and AarI are active in ligate buffer, but for AarI the...
accompanying oligonucleotides were supplemented for optimal efficiency. For BbsI half of the ligase buffer was replaced by NEBuffer 2.1 and for BsmBI half was replaced by NEBuffer 3.1.

The general assembly protocol was 37 °C, 30 min; 16 °C, 30 min; (37 °C, 3 min; 16 °C, 5 min) × 15; 37 °C, 10 min; 50 °C, 10 min; 80 °C, 10 min. The exception was BsmBI, where 55 °C were required for the first incubation step and ligase was only added afterwards. 7.5 µl of the final reaction were used for E. coli transformation.

Measurement of \( P_{\text{sysA}} \) activity. Fluorescence intensity of strains carrying a transcriptional fusion of the xylose-inducible promoter \( P_{\text{sysA}} \) and the fluorescence reporter \( mKate2 \) were assayed using a Synergy™ NEOALPHAB multi-mode microplate reader from BioTek© (Winooski, VT, USA). The reader was controlled using the software Gen5™ (version 2.06). Cells were inoculated 1:1000 from overnight cultures and grown to OD\(_{600} \) ~0.2, treated with 0.2% xylose and grown for two hours. Cells were harvested by centrifugation and resuspended in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na\(_2\)HPO\(_4\), 1.8 mM KH\(_2\)PO\(_4\), pH 7.4). 200 µl per well in 96-well plates (black walls, clear bottom; Greiner Bio-One, Frickenhausen, Germany) were measured for their OD\(_{600} \) and mKate-fluorescence using the monochrometer with following parameters: endpoint measurement, gain: 100, excitation wavelength: 588 nm, emission wavelength: 633 nm.

Data availability. The vectors generated in this study are available from the Bacillus Genetic Stock Center (BGSC, http://www.bgsc.org/, accession numbers ECE701-26) and the SEVA collection (http://www.user.cnb.csic.es/~seva/#page_id=19). For sequence information, the following accession numbers apply: GenBank, KY995178 to KY995203; ACS Synthetic Biology Registry (https://acs-registry.iberi.org/); JPU/008862 to JPU/008887.

References

1. Wright, O., Delmans, M., Stan, G. B. & Ellis, T. GeneGuard: A modular plasmid system designed for biosafety. ACS Synth Biol 4, 307–316, https://doi.org/10.1021/sb500234a (2015).
2. Shimada, T. et al. Classification and strength measurement of stationary-phase promoters by use of a newly developed promoter cloning vector. J Bacteriol 186, 7112–7122, https://doi.org/10.1128/JB.186.21.7112-7122.2004 (2004).
3. Arnaud, M., Chastanet, A. & Debbarbouille, M. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. Appl Environ Microbiol 70, 6887–6891, https://doi.org/10.1128/AEM.70.11.6887-6891.2004 (2004).
4. Sinha, N., Williams, C. A., Piper, R. C. & Shields, S. B. A set of dual promoter vectors for high throughput cloning, screening, and protein expression in eukaryotic and prokaryotic systems from a single plasmid. BMC Biotechnol 12, 54, https://doi.org/10.1186/1472-6750-12-54 (2012).
5. Andrianantoandro, E., Basu, S., Karig, D. K. & Weiss, R. Synthetic biology: new engineering rules for an emerging discipline. Mol Syst Biol 2(2006), 0028, https://doi.org/10.1038/msb4100071 (2006).
6. Martinez-Garcia, E., Aparricio, T., Goni-Moreno, A., Fraile, S. & de Lorenzo, V. SEVA 2.0: an update of the Standard European Vector Architecture for de-/re-construction of bacterial functionalities. Nucleic Acids Res 43, D1183–1189, https://doi.org/10.1093/nar/gku1114 (2015).
7. Silva-Rocha, R. et al. The Standard European Vector Architecture (SEVA): a coherent platform for the analysis and deployment of complex prokaryotic phenotypes. Nucleic Acids Res 41, D666–675, https://doi.org/10.1093/nar/gku1119 (2013).
8. Standard European Vector Architecture. http://www.user.cnb.csic.es/~seva/#page_id=17 and http://www.user.cnb.csic.es/~seva/#page_id=19 (accessed March 2017).
9. Kim, S. H., Cavaleiro, A. M., Rennig, M. & Norholm, M. H. SEVA Linkers: A versatile and automatable DNA backbone exchange standard for synthetic biology. ACS Synth Biol 5, 1177–1181, https://doi.org/10.1021/acssynbio.5b00257 (2016).
10. van Diil, J. M. & Hecker, M. Bacillus subtilis: from soil bacterium to super-secreting cell factory. Microb Cell Fact 12, 3, https://doi.org/10.1186/1475-2859-12-3 (2013).
11. Hohmann, H.-P., van Diil, J. M., Krishnappa, L. & Pragai, Z. In Industrial Biotechnology, 221–297 (Wiley-VCH Verlag GmbH & Co. KGAA, 2017).
12. Liu, Y., Li, J., Du, G., Chen, J. & Liu, L. Metabolic engineering of Bacillus subtilis fueled by systems biology: Recent advances and future directions. Biotechnol Adv 35, 20–30, https://doi.org/10.1016/j.biotechadv.2017.01.003 (2017).
13. Mell, J. C. & Redfield, R. J. Natural competence and the evolution of DNA uptake specificity. J Bacteriol 196, 1471–1483, https://doi.org/10.1128/JB.01293-13 (2014).
14. Zucca, S., Pasotti, L., Mazzini, G., DeAngelis, M. G. & Magni, P. Characterization of an inducible promoter in different DNA copy number conditions. BMC Biotechnol 13(Suppl 4), S11, https://doi.org/10.1186/1471-2105-13-S4-S11 (2012).
15. Adams, C. W. & Hatfield, G. W. Effects of Promoter Strengths and Growth Conditions on Copy Number of Transcription-Fusion Vectors. J Biol Chem 259, 7399–7403 (1984).
16. Guerout-Fleury, A. M., Frandsen, N. & Stragier, P. Plasmids for ectopic integration in Bacillus subtilis. Gene 180, 57–61 (1996).
17. Radeck, J. et al. The Bacillus BioBrick Boc: generation and evaluation of essential genetic building blocks for standardized work with Bacillus subtilis. J Biol Eng 7, 29, https://doi.org/10.1186/1754-1611-7-29 (2013).
18. Hartl, B., Wehl, W., Wiegert, T., Homuth, G. & Schumann, W. Development of a new integration site within the Bacillus subtilis chromosome and construction of compatible expression cassettes. J Bacteriol 183, 2696–2699, https://doi.org/10.1128/JB.183.8.2696-2699.2001 (2001).
19. Schmalisch, M. et al. Small genes under sporulation control in the Bacillus subtilis genome. J Bacteriol 192, 5402–5412, https://doi.org/10.1128/JB.00534-10 (2010).
20. Sollik, J. et al. Induction of the Bacillus subtilis ptsGHI operon by glucose is controlled by a novel antiterminator, GlcT. Mol Microbiol 25, 65–78 (1997).
21. Wach, A. PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in S. cerevisiae. Yeast 12, 259–265 (1996).
22. Harwood, C. R. & Cutting, S. M. Molecular Biological Methods for Bacillus (John Wiley & Sons, 1990).
23. Engler, C., Kandzia, R. & Marillonnet, S. A one pot, one step, precision cloning method with high throughput capability. PLoS One 3, e3647, https://doi.org/10.1371/journal.pone.0003647 (2008).
24. Engler, C., Gruetzner, R., Kandzia, R. & Marillonnet, S. Golden gate shuffling: a one-pot DNA shuffling method based on type IIs restriction enzymes. PLoS One 4, e5553, https://doi.org/10.1371/journal.pone.0005553 (2009).
25. Khasanov, F. K., Zvingila, D. J., Zainullin, A. A., Prozorov, A. A. & Bashkirov, V. I. Homologous recombination between plasmid and chromosomal DNA in Bacillus subtilis requires approximately 70 bp of homology. Mol Gen Genet 234, 494–497, https://doi.org/10.1007/bf00538711 (1992).
26. Gibson, D. G. Synthesis of DNA fragments in yeast by one-step assembly of overlapping oligonucleotides. Nucleic Acids Res 37, 6984–6990, https://doi.org/10.1093/nar/gkp687 (2009).
27. Gibson, D. G., Smith, H. O., Hutchinson, C. A. 3rd, Venter, J. C. & Merryman, C. Chemical synthesis of the mouse mitochondrial genome. Nat Methods 7, 901–903, https://doi.org/10.1038/nmeth.1515 (2010).
28. Weber, E., Engler, C., Gruetzner, R., Werner, S. & Marillonnet, S. A modular cloning system for standardized assembly of multigene constructs. PLoS ONE 6, e16765, https://doi.org/10.1371/journal.pone.0016765 (2011).
29. Sarrion-Perdigones, A. et al. GoldenBraid: An iterative cloning system for standardized assembly of reusable genetic modules. PLoS ONE 6, e21622, https://doi.org/10.1371/journal.pone.0021622 (2011).
30. Sarrion-Perdigones, A. et al. GoldenBraid 2.0: a comprehensive DNA assembly framework for plant synthetic biology. Plant Physiol 162, 1618–1631, https://doi.org/10.1104/pp.113.217661 (2013).
31. Sauer, C. et al. Effect of genome position on heterologous gene expression in Bacillus subtilis: An unbiased analysis. ACS Synthetic Biology 5, 942–947, https://doi.org/10.1021/acssynbio.6b00065 (2016).
32. Turan, S., Zehe, C., Koeble, J., Qiao, J. & Boe, J. Recombinase-mediated cassette exchange (RMCE) - a rapidly-expanding toolbox for targeted genomic modifications. Gene 515, 1–27, https://doi.org/10.1016/j.gene.2012.11.016 (2013).
33. Dong, H. & Zhang, D. Current development in genetic engineering strategies of Bacillus species. Microb Cell Fact 13, 63, https://doi.org/10.1186/1475-2859-13-63 (2014).
34. Martinez-Garcia, E., Calles, B., Arevalo-Rodriguez, M. & de Lorenzo, V. pBAM1: an all-synthetic genetic tool for analysis and construction of complex bacterial phenotypes. BMC Microbiol 11, 38, https://doi.org/10.1186/1471-2180-11-38 (2011).
35. Trieu-Cuot, P., Carlier, C., Martin, P. & Courvalin, P. Plasmid transfer by conjugation from Escherichia coli to Gram-positive bacteria. FEMS Microbiol Lett 48, 289–294, https://doi.org/10.1111/j.1574-6968.1987.tb02558.x (1987).
36. Vagner, V. & Ehrlich, S. D. Efficiency of homologous DNA recombination varies along the Bacillus subtilis chromosome. J Bacteriol 170, 3978–3982 (1988).
37. Rachinger, M. et al. Size unlimited markerless deletions by a transconjugative plasmid-system in Bacillus licheniformis. J Biotechnol 167, 365–369, https://doi.org/10.1016/j.jbiotec.2013.07.026 (2013).
38. Mühlhardt, C. Der Experimentator. Molekularbiologie/Genomik. 6 edn, (Spektrum Akademischer Verlag, 2009).
39. Sambrook, J. & Russell, D. W. Molecular Cloning - a laboratory manual (Cold Spring Harbor Laboratory Press, 2001).
40. Joseph, P., Fantino, J. R., Herbaud, M. L. & Denizot, F. Rapid oriented cloning in a shuttle vector allowing modulated gene expression in Bacillus subtilis. FEMS Microbiol Lett 205, 91–97 (2001).
41. Guerout-Fleury, A. M., Shazand, K., Frandsen, N. & Stragier, P. Antibiotic-resistance cassettes for Bacillus subtilis. Gene 167, 335–336 (1995).
42. Balbás, P. & Bolívar, F. In Recombinant Gene Expression: Reviews and Protocols (eds Paulina Balbás & Argelia Lorence) 77–90 (Humana Press, 2004).

Acknowledgements
We thank Wilfried Meijer for fruitful initial design discussions and Beate Lüdke for assistance with B. subtilis transformations. This work was supported by the Deutsche Forschungsgemeinschaft (DFG) priority program SPP1617 ‘Phenotypic Heterogeneity and Sociobiology of Bacterial Populations’ [grant number MA 2837/3-2 to TM]. Funding for open access charge: dito.

Author Contributions
J.R. and T.M. conceptualized Bacillus SEV A siblings. J.R. detailed and planned the experiments, analyzed the data and created the figures. J.R., D.M. and N.L. cloned the vectors and J.R. performed Golden Gate assemblies. J.R. and T.M. wrote the manuscript. All authors reviewed the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-14329-5.

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

Change History: A correction to this article has been published and is linked from the HTML version of this paper. The error has been fixed in the paper.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017