Abstract:

Title: BACTERIAL MUTANTS WITH IMPROVED TRANSFORMATION EFFICIENCY

Provided herein are Bacillus mutants having improved transformation efficiency, comprising a disruption to an endogenous mecA gene and disruption to an endogenous sirA gene. Also described are methods for producing the mutants, methods for generating transformants using the mutants, and methods for producing a polypeptide or fermentation product using the mutants.
BACTERIAL MUTANTS WITH IMPROVED TRANSFORMATION EFFICIENCY

Reference to a Sequence Listing

This application contains a Sequence Listing in computer readable form, which is incorporated herein by reference.

Background

Genetic competence is a physiological state in which exogenous DNA can be internalized, leading to a transformation event (Berka et al., Mol. Microbiol. 2002, 43,1331-1345), but is distinct from artificial transformation involving electroporation, protoplasts, and heat shock or CaCl₂ treatment. Natural competence has been observed in both Gram positive and Gram negative bacterial species (Dubnau, Annual Rev. Microbiol. 1999, 53, 217-244), and the process requires more than a dozen proteins whose expression is precisely choreographed to the needs of each organism.

Several hypotheses have been proposed regarding the purpose of natural competence, and they can be summarized as DNA for food, DNA for repair, and DNA for genetic diversity (Dubnau, 1999, supra). The DNA for food hypothesis is supported by observations that competence is a stationary phase phenomenon that occurs when cells are nutrient limited, and often a powerful nonspecific nuclease is co-expressed with transformation specific proteins. Evidence for the second hypothesis comes from the fact that genes encoding DNA repair enzymes are coordinately expressed with those encoding DNA transport proteins. Lastly, the DNA for genetic diversity hypothesis proposes that competence is a mechanism for exploring the fitness landscape via horizontal gene transfer. Observations that competence is regulated by a quorum-sensing mechanism and that it is a bistable condition (Avery, Trends Microbiol. 2005, 13, 459-462) support this hypothesis.

Public databases now contain a multitude of complete bacterial genomes, including several genomes from different strains of the same species. Recent analyses have shown, using pairwise whole genome alignments, that different strains of the same species may vary substantially in gene content. For example, genome comparisons of Escherichia coli strains CFT073, EDL933, and MG1655 revealed that only 39.2% of their combined set of proteins (gene products) are common to all three strains, highlighting the astonishing diversity among strains of the same species (Blattner et al., Science 1997, 277, 1453-1474; Hayashi et al., 2006, Mol. Syst. Biol. doi:10.1038:msb4100049; Perna et al., Nature 2001, 409, 529-533; Welch et al., Proc. Natl. Acad. Sci. USA 2002, 99, 17020-17024). Furthermore, the genome sequence of E. coli strain CFT073 revealed 1,623 strain-specific genes (21.2%). From comparisons of this type, it is clearly seen that bacterial genomes are
segmented into a common conserved backbone and strain-specific sequences. Typically
the genome of a given strain within a species shows a mosaic structure in terms of the
distribution of conserved "backbone" genes conserved among all strains and non-conserved
genes that may have been acquired by horizontal transfer (Brzuszkiewicz et al., Proc. Natl.
Acad. Sci. USA 2006, 103, 12879-12884; Welch et al., 2002, supra).

In terms of practical utility, transformation via natural competence is an extremely
useful tool for constructing bacterial strains, e.g., Bacillus, that may contain altered alleles
for chromosomal genes or plasmids assembled via recombinant DNA methods. Although
transformation of certain species with plasmids and chromosomal DNA may be achieved via
artificial means as noted above (e.g., electroporation, protoplasts, and heat shock or CaCl₂
treatment), introduction of DNA by natural competence offers clear advantages of simplicity,
convenience, speed, and efficiency.

In Bacillus subtilis, only 5-10% of the cells in a population differentiate to a
competent state (termed the K-state) via a process that involves quorum-sensing, signal
transduction, and a cascade of gene expression (Avery, 2005, supra). At least 50 genes are
known to be involved directly in competence, and as many as 165 genes are regulated
(directly or indirectly) by the central transcription factor ComK (Berka et al., 2002, supra).
The competence cascade in Bacillus subtilis consists of two regulatory modules punctuated
by a molecular switch (Figure 1) that involves ComS binding to the adaptor molecule MecA,
thereby interfering with degradation of the transcription factor ComK by the ClpC/ClpP
protease (Turgay et al., EMBO J. 1998, 17, 6730-6738).

In Bacillus subtilis, mecA inactivation has been shown to moderately elevate
transformation efficiency due to increased availability of ComK (Hahn et. al, Mol. Microbiol.,
1995, 18, 755-767). A recent report suggests a Bacillus subtilis mecA deletion results in an
increased expression of the eps and tasA operons (Prepiak et. al, Mol. Microbiol., 2011, 80,
1014-1030) in accordance with the regulatory relationship between mecA and the eps and
tasA regulons shown in Figure 2.

With the exception of comP and comS, Bacillus licheniformis harbors orthologues of
the genes necessary to achieve natural competence. Ostensibly naturally competent
Bacillus licheniformis cells cannot be obtained due to the lack of a functional comS gene
resulting in ComK continually sequestered by MecA, and proteolytically degraded by
ClpC/P/MecA complex. Applicants have shown that expression of ComS and ComK in
Bacillus licheniformis can improve competence (US201 0/0028944).

Since Bacillus species provide a key platform for a variety of industrially relevant
processes, such as metabolic engineering and biochemical production, engineering strains
that manifest improved competence is highly desirable for construction of new and improved
production strains. The availability of a turn-key method for improving competence in *Bacillus* strains would improve the speed and efficiency with which chromosomal markers/alleles and expression vectors could be introduced. The present invention fulfills these and other needs.

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**Summary**

Described herein are *Bacillus* mutants having improved transformation efficiency. The Applicants have surprisingly found that disruption to both endogenous genes *mecA* and *sinl* in a *Bacillus* host cell shows significantly improved transformation efficiency compared to disruption of the endogenous *mecA* gene alone.

In one aspect is a mutant of a parent *Bacillus* strain, comprising disruption to an endogenous *mecA* gene and disruption to an endogenous *sinl* gene, wherein the mutant has improved transformation efficiency compared to the parent *Bacillus* strain that lacks disruption to the endogenous *mecA* gene and lacks disruption to the endogenous *sinl* gene, when cultivated under identical conditions. In some aspects, the *Bacillus* mutant is a *Bacillus licheniformis* mutant or a *Bacillus subtilis* mutant.

Also described are methods for obtaining the *Bacillus* mutants, comprising disrupting in a parent *Bacillus* strain comprising disrupting in a parent *Bacillus* strain an endogenous *mecA* gene and an endogenous *sinl* gene.

Also described are methods for obtaining a *Bacillus* transformant, comprising transforming a heterologous polynucleotide into the *Bacillus* mutant.

Also described are methods of producing a polypeptide, comprising: (a) cultivating a *Bacillus* transformant comprising a heterologous polynucleotide encoding the polypeptide; and (b) recovering the polypeptide.

Also described are methods of producing a fermentation product, comprising: (a) cultivating the *Bacillus* transformant comprising a heterologous polynucleotide encoding a polypeptide of the fermentation pathway; and (b) recovering the fermentation product.

**Brief Description of the Figures**

Figure 1 shows the competence regulatory cascade of *Bacillus subtilis*. Module 1 involves detection of the competence pheromone CSF and signal transduction via a phosphorelay mechanism resulting in synthesis of the ComS peptide. ComS interferes with proteolytic degradation of the transcription factor ComK via binding to MecA that activates Module 2 encoding the late competence functions encoding DNA transport machinery.

Figure 2 shows the regulatory relationship between *mecA* and the *eps* and *tasA* regulons in *Bacillus subtilis*. 
Figure 3 shows the DNA sequence and the deduced amino acid sequence of the *Bacillus licheniformis meCA* gene (SEQ ID NOs: 1 and 2, respectively).

Figure 4 shows the DNA sequence and the deduced amino acid sequence of the *Bacillus licheniformis sinl* gene (SEQ ID NOs: 3 and 4, respectively).

Figure 5 shows the DNA sequence and the deduced amino acid sequence of the *Bacillus subtilis meCA* gene (SEQ ID NOs: 15 and 16, respectively).

Figure 6 shows the DNA sequence and the deduced amino acid sequence of the *Bacillus subtilis sinl* gene (SEQ ID NOs: 17 and 18, respectively).

Figure 7 shows the transformation efficiency of strains TaHy9 comprising a *meCA* gene disruption.

Figure 8 shows the relative transformation efficiency of strains TaHy9 (comprising a *meCA* gene disruption) and BaC0155 (comprising a *meCA* gene disruption and a *sinl* gene disruption).

**Definitions**

**Disruption:** The term “disruption” means that a coding region and/or control sequence of a referenced gene is partially or entirely modified (such as by deletion, insertion, and/or substitution of one or more nucleotides, or by association with RNAi or antisense technology) resulting in the absence (inactivation) or decrease in expression, and/or the absence or decrease of enzyme activity of the encoded polypeptide. The effects of disruption can be measured using techniques known in the art such as detecting the absence or decrease of enzyme activity using from cell-free extract measurements referenced herein; or by the absence or decrease of corresponding mRNA (e.g., at least 25% decrease, at least 50% decrease, at least 60% decrease, at least 70% decrease, at least 80% decrease, or at least 90% decrease); the absence or decrease in the amount of corresponding polypeptide having enzyme activity (e.g., at least 25% decrease, at least 50% decrease, at least 60% decrease, at least 70% decrease, at least 80% decrease, or at least 90% decrease); or the absence or decrease of the specific activity of the corresponding polypeptide having enzyme activity (e.g., at least 25% decrease, at least 50% decrease, at least 60% decrease, at least 70% decrease, at least 80% decrease, or at least 90% decrease). Disruptions of a particular gene of interest can be generated by methods known in the art, e.g., by directed homologous recombination (see *Methods in Yeast Genetics* (1997 edition), Adams, Gottschling, Kaiser, and Stems, Cold Spring Harbor Press (1998)). Techniques to disrupt *Bacillus* genes are described herein and have been demonstrated in the art (see Stahl & Ferrari, *J. Bacteriol*. 1984, 158, 41-1418).
Parent: The term "parent" or "parent Bacillus strain" means a Bacillus strain to which a disruption is made to produce a mutant Bacillus strain described herein. The parent may be a naturally occurring (wild-type) or previously modified Bacillus strain.

Mutant: The term "mutant" means the resulting Bacillus strain after one or more disruptions are made to a parent Bacillus strain.

Improved transformation efficiency: The term "improved transformation efficiency" means that the referenced Bacillus mutant strain is capable of generating an increased number of transformants compared to the parent Bacillus strain when transformed and cultivated under identical conditions. Improved transformation efficiency can be demonstrated by generating an increased number of transformants using transformation methods described in the Examples below. Improved transformation efficiency may also be demonstrated using methods previously described (e.g., Anagnostopoulos and Spizizen, J. Bacteriol. 1961, 81, 741-746). In some aspects, the Bacillus mutant strain is capable of producing at least 2-fold, e.g., at least 5-fold, at least 10-fold, at least 20-fold, at least 50-fold, at least 100-fold, at least 200-fold, at least 500-fold, at least 1000-fold, at least 2000-fold, at least 5000-fold, at least 10000-fold, at least 20000-fold, at least 50000-fold, or at least 100000-fold more transformants compared to the parent Bacillus strain that lacks disruption to the endogenous mecA gene and lacks disruption to the endogenous sinl gene, when cultivated under identical conditions.

Coding sequence: The term "coding sequence" means a polynucleotide sequence, which specifies the amino acid sequence of a polypeptide. The boundaries of the coding sequence are generally determined by an open reading frame, which usually begins with the ATG start codon or alternative start codons such as GTG and TTG and ends with a stop codon such as TAA, TAG, and TGA. The coding sequence may be a sequence of genomic DNA, cDNA, a synthetic polynucleotide, and/or a recombinant polynucleotide.

Sequence Identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "sequence identity".

For purposes described herein, the degree of sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, J. Mol. Biol. 1970, 48, 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., Trends Genet 2000, 16, 276-277), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows:
(Identical Residues x 100)/(Length of Alignment - Total Number of Gaps in Alignment)

For purposes described herein, the degree of sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, supra) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, supra), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS version of NCBI NUC4.4) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows:

(Identical Deoxyribonucleotides x 100)/(Length of Alignment - Total Number of Gaps in Alignment)

Hybridization conditions: The term "very low stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 45°C.

The term "low stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 25% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 50°C.

The term "medium stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 55°C.

The term "medium-high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 35% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 60°C.

The term "high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following
standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 65°C.

The term "very high stringency conditions" means for probes of at least 100 nucleotides in length, prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/ml sheared and denatured salmon sperm DNA, and 50% formamide, following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed three times each for 15 minutes using 0.2X SSC, 0.2% SDS at 70°C.

**Heterologous polynucleotide:** The term "heterologous polynucleotide" is defined herein as a polynucleotide that is not native to the host cell; a native polynucleotide in which one or more (e.g., two, several) structural modifications have been made to the coding region; a native polynucleotide whose expression is quantitatively altered as a result of manipulation of the DNA by recombinant DNA techniques, e.g., a different (foreign) promoter linked to the polynucleotide; or a native polynucleotide whose expression is quantitatively altered by the introduction of one or more extra copies of the polynucleotide into the host cell.

**Isolated:** The term "isolated" means a substance in a form or environment that does not occur in nature. Non-limiting examples of isolated substances include (1) any non-naturally occurring substance, (2) any substance including, but not limited to, any host cell, enzyme, variant, nucleic acid, protein, peptide or cofactor, that is at least partially removed from one or more or all of the naturally occurring constituents with which it is associated in nature; (3) any substance modified by the hand of man relative to that substance found in nature; or (4) any substance modified by increasing the amount of the substance relative to other components with which it is naturally associated.

**Endogenous gene:** The term "endogenous gene" means a gene that is native to the parent *Bacillus* strain.

**Nucleic acid construct:** The term "nucleic acid construct" means a polynucleotide comprises one or more (e.g., two, several) control sequences. The polynucleotide may be single-stranded or double-stranded, and may be isolated from a naturally occurring gene, modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature, or synthetic.

**Control sequence:** The term "control sequence" means a nucleic acid sequence necessary for polypeptide expression. Control sequences may be native or foreign to the polynucleotide encoding the polypeptide, and native or foreign to each other. Such control sequences include, but are not limited to, a leader sequence, polyadenylation sequence, propeptide sequence, promoter sequence, signal peptide sequence, and transcription terminator sequence. The control sequences may be provided with linkers for the purpose of
introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the polynucleotide encoding a polypeptide.

**Operably linked:** The term "operably linked" means a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of a polynucleotide such that the control sequence directs the expression of the coding sequence.

**Expression:** The term "expression" includes any step involved in the production of the polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion. Expression can be measured—for example, to detect increased expression—by techniques known in the art, such as measuring levels of mRNA and/or translated polypeptide.

**Expression vector:** The term "expression vector" means a linear or circular DNA molecule that comprises a polynucleotide encoding a polypeptide and is operably linked to control sequences, wherein the control sequences provide for expression of the polynucleotide encoding the polypeptide. At a minimum, the expression vector comprises a promoter sequence, and transcriptional and translational stop signal sequences.

**Host cell:** The term "host cell" means any cell type that is susceptible to transformation, transfection, transduction, and the like with a nucleic acid construct or expression vector. The term "host cell" encompasses any progeny of a parent cell that is not identical to the parent cell due to mutations that occur during replication.

**Transformation:** The term "transformation" means introducing a heterologous polynucleotide into a *Bacillus* cell so that the DNA is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector. The resulting *Bacillus* cell following transformation is described herein as a "transformant."

**Allelic variant:** The term "allelic variant" means any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

Reference to "about" a value or parameter herein includes aspects that are directed to that value or parameter *per se*. For example, description referring to "about X" includes the aspect "X". When used in combination with measured values, "about" includes a range that encompasses at least the uncertainty associated with the method of measuring the particular value, and can include a range of plus or minus two standard deviations around the stated value.
As used herein and in the appended claims, the singular forms "a," "or," and "the" include plural referents unless the context clearly dictates otherwise. It is understood that the aspects described herein include "consisting" and/or "consisting essentially of" aspects.

Unless defined otherwise or clearly indicated by context, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art.

**Detailed Description**

**Bacillus Mutants**

Described herein, *inter alia*, are mutants of a parent *Bacillus* strain, comprising a disruption to an endogenous *mecA* gene and disruption to an endogenous *sinl* gene, wherein the mutant has improved transformation efficiency.

The parent strain of the mutants and related methods may be any *Bacillus* strain, such as a wild-type *Bacillus* or a mutant thereof. In some aspects, the parent *Bacillus* strain is a *Bacillus* alkalophilus, *Bacillus* amyoliquefaciens, *Bacillus* brevis, *Bacillus* circulans, *Bacillus* clausii, *Bacillus* coagulans, *Bacillus* firmus, *Bacillus* lautus, *Bacillus* lentus, *Bacillus* licheniformis, *Bacillus* megaterium, *Bacillus* pumilus, *Bacillus* stearothermophilus, *Bacillus* subtilis, or *Bacillus* thuringiensis strain.

The disrupted gene may be any suitable endogenous *Bacillus meca* gene and *sinl* gene. Examples of target genes include the *Bacillus licheniformis meca* gene of SEQ ID NO: 1 (encoding the polypeptide of SEQ ID NO: 2), the *Bacillus licheniformis sinl* gene of SEQ ID NO: 3 (encoding the polypeptide of SEQ ID NO: 4), *Bacillus subtilis meca* gene of SEQ ID NO: 15 (encoding the polypeptide of SEQ ID NO: 16), and the *Bacillus subtilis sinl* gene of SEQ ID NO: 17 (encoding the polypeptide of SEQ ID NO: 18).

In some aspects of the mutants and related methods, the endogenous *mecA* gene encodes for a polypeptide having at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or 100% sequence identity to SEQ ID NO: 2 or 16. In some aspects, the endogenous *mecA* gene encodes for a polypeptide having a sequence that differs by no more than ten amino acids, e.g., by no more than five amino acids, by no more than four amino acids, by no more than three amino acids, by no more than two amino acids, or by one amino acid from SEQ ID NO: 2 or 16. In some aspects, the endogenous *mecA* gene encodes for a polypeptide comprising or consisting of SEQ ID NO: 2 or 16.

In other aspects of the mutants or related methods, the endogenous *mecA* gene comprises a coding sequence that has at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least
99%, or 100% sequence identity to SEQ ID NO: 1 or 15. In some aspects, the coding sequence of the endogenous meca gene comprises or consists of SEQ ID NO: 1 or 15.

In other aspects of the mutants or related methods, the endogenous meca gene comprises a coding sequence that hybridizes under at least low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with the full-length complementary strand of SEQ ID NO: 1 or 15.

In some aspects of the mutants and related methods, the endogenous sinl gene encodes for a polypeptide having at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 4 or 18. In some aspects, the endogenous sinl gene encodes for a polypeptide having a sequence that differs by no more than ten amino acids, e.g., by no more than five amino acids, by no more than four amino acids, by no more than three amino acids, by no more than two amino acids, or by one amino acid from SEQ ID NO: 4 or 18. In some aspects, the endogenous sinl gene encodes for a polypeptide comprising or consisting of SEQ ID NO: 4 or 18.

In other aspects of the mutants or related methods, the endogenous sinl gene comprises a coding sequence that has at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 3 or 17. In some aspects, the coding sequence of the endogenous sinl gene comprises or consists of SEQ ID NO: 3 or 17.

In other aspects of the mutants or related methods, the endogenous sinl gene comprises a coding sequence that hybridizes under at least low stringency conditions, medium stringency conditions, medium-high stringency conditions, high stringency conditions, or very high stringency conditions with the full-length complementary strand of SEQ ID NO: 3 or 17.

The polynucleotide sequences disclosed herein, or a subsequences thereof; as well as the amino acid sequences described herein, of or a fragment thereof; may be used to design nucleic acid probes to identify and clone homologous meca and sinl genes from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the DNA from a Bacillus species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, e.g., at least 14 nucleotides, at least 25 nucleotides, at least 35 nucleotides, at least 70 nucleotides in lengths. The probes may be longer, e.g., at least 100 nucleotides, at least 200 nucleotides, at least 300 nucleotides, at least 400 nucleotides, at least 500
nucleotides in lengths. Even longer probes may be used, e.g., at least 600 nucleotides, at least 700 nucleotides, at least 800 nucleotides, or at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with \(^{32}\)P, \(^{3}H\), \(^{35}\)S, biotin, or avidin).

A DNA library prepared from such other strains may be screened for DNA that hybridizes with the probes described above. Genomic or other DNA from such other strains may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA that is homologous with the polynucleotide sequences described herein, or a subsequence thereof, the carrier material may be used in a Southern blot. For purposes of the probes described above, hybridization indicates that the polynucleotide hybridizes to a labeled nucleic acid probe corresponding to the polynucleotide sequences, the full-length complementary strand thereof, or a subsequence of the foregoing; under very low to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions can be detected using, for example, X-ray film.

For long probes of at least 100 nucleotides in length, very low to very high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 micrograms/mL sheared and denatured salmon sperm DNA, and either 25% formamide for very low and low stringencies, 35% formamide for medium and medium-high stringencies, or 50% formamide for high and very high stringencies, following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS at 45°C (very low stringency), at 50°C (low stringency), at 55°C (medium stringency), at 60°C (medium-high stringency), at 65°C (high stringency), and at 70°C (very high stringency).

For short probes of about 15 nucleotides to about 70 nucleotides in length, stringency conditions are defined as prehybridization and hybridization at about 5°C to about 10°C below the calculated \(T_m\) using the calculation according to Bolton and McCarthy (Proc. Natl. Acad. Sci. USA 1962, 48, 1390) in 0.9 M NaCl, 0.09 M Tris-HCl pH 7.6, 6 mM EDTA, 0.5% NP-40, 1X Denhardt’s solution, 1 mM sodium pyrophosphate, 1 mM sodium monobasic phosphate, 0.1 mM ATP, and 0.2 mg of yeast RNA per mL following standard Southern blotting procedures for 12 to 24 hours optimally. The carrier material is finally washed once in 6X SSC plus 0.1% SDS for 15 minutes and twice each for 15 minutes using 6X SSC at 5°C to 10°C below the calculated \(T_m\).
changes that are of a minor nature, that is conservative amino acid substitutions or 
insertions that do not significantly affect the folding and/or activity of the protein; small 
deletions, typically of one to about 30 amino acids; small amino-terminal or carboxyl-terminal 
extensions, such as an amino-terminal methionine residue; a small linker peptide of up to 
about 20-25 residues; or a small extension that facilitates purification by changing net 
charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding 
domain.

Examples of conservative substitutions are within the group of basic amino acids 
(arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar 
amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and 
valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino 
acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions that do 
not generally alter specific activity are known in the art and are described, for example, by 
commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, 
Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and 
Asp/Gly.

Essential amino acids can be identified according to procedures known in the art, 
such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and 
Wells, Science 1989, 244, 1081-1085). In the latter technique, single alanine mutations are 
introduced at every residue in the molecule, and the resultant mutant molecules are tested 
for activity to identify amino acid residues that are critical to the activity of the molecule. See 
also, Hilton et al., J. Biol. Chem. 1996, 271, 4699-4708. The active site of the enzyme or 
other biological interaction can also be determined by physical analysis of structure, as 
determined by such techniques as nuclear magnetic resonance, crystallography, electron 
diffraction, or photoaffinity labeling, in conjunction with mutation of putative contact site 
amino acids. See, for example, de Vos et al., Science 1992, 255, 306-312; Smith et al., J. 
of essential amino acids can also be inferred from analysis of identities with other related 
enzymes.

Single or multiple amino acid substitutions, deletions, and/or insertions can be made 
and tested using known methods of mutagenesis, recombination, and/or shuffling, followed 
by a relevant screening procedure, such as those disclosed by Reidhaar-Olson and Sauer, 
2156; WO 95/17413; or WO 95/22625. Other methods that can be used include error-prone 
PCR, phage display (e.g., Lowman et al., Biochemistry 1991, 30, 10832-10837; U.S. Patent
No. 5,223,409; WO 92/06204), and region-directed mutagenesis (Derbyshire et al., Gene 1986, 46, 145; Ner et al., DNA 1988, 7, 127).

Mutagenesis/shuffling methods can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides expressed by host cells (Ness et al., Nature Biotechnol. 1999, 17, 893-896). Mutagenized DNA molecules that encode active enzymes can be recovered from the host cells and rapidly sequenced using standard methods in the art. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide.

**Disruption of mecA and sinl Genes and Methods of Producing Bacillus Mutants**

The Bacillus mutant strains described herein may be constructed by disrupting the referenced endogenous mecA and sinl genes using methods well known in the art, including those methods described herein. A portion of the gene can be disrupted such as the coding region or a control sequence required for expression of the coding region. Such a control sequence of the gene may be a promoter sequence or a functional part thereof, i.e., a part that is sufficient for affecting expression of the gene. For example, a promoter sequence may be inactivated resulting in no expression or a weaker promoter may be substituted for the native promoter sequence to reduce expression of the coding sequence. Other control sequences for possible modification include, but are not limited to, a leader, propeptide sequence, signal sequence, transcription terminator, and transcriptional activator.

The Bacillus mutant strains may be constructed by gene deletion techniques to eliminate or reduce expression of the gene. Gene deletion techniques enable the partial or complete removal of the gene thereby eliminating expression. In such methods, deletion of the gene is accomplished by homologous recombination using a plasmid that has been constructed to contiguously contain the 5' and 3' regions flanking the gene.

The Bacillus mutant strains may also be constructed by introducing, substituting, and/or removing one or more (e.g., two, several) nucleotides in the gene or a control sequence thereof required for the transcription or translation thereof. For example, nucleotides may be inserted or removed for the introduction of a stop codon, the removal of the start codon, or a frame-shift of the open reading frame. Such a modification may be accomplished by site-directed mutagenesis or PCR generated mutagenesis in accordance with methods known in the art. See, for example, Botstein and Shortle, Science 1985, 229, 4719; Lo et al., Proc. Natl. Acad. Sci. U.S.A. 1985, 81, 2285; Higuchi et al., Nucleic Acids Res 1988, 16, 7351; Shimada, Meth. Mol. Biol. 1996, 57, 157; Ho et al., Gene 1989, 77, 61; Horton et al., Gene 1989, 77, 61; and Sarkar and Sommer, BioTechniques 1990, 8, 404.

The Bacillus mutant strains may also be constructed by gene disruption techniques
by inserting into the gene a disruptive nucleic acid construct comprising a nucleic acid
fragment homologous to the gene that will create a duplication of the region of homology
and incorporate construct DNA between the duplicated regions. Such a gene disruption can
eliminate gene expression if the inserted construct separates the promoter of the gene from
the coding region or interrupts the coding sequence such that a non-functional gene product
results. A disrupting construct may be simply a selectable marker gene accompanied by 5’
and 3’ regions homologous to the gene. The selectable marker enables identification of
transformants containing the disrupted gene.

The *Bacillus* mutant strains may also be constructed by the process of gene
conversion (see, for example, Iglesias and Trautner, *Molecular General Genetics* 1983, 189,
73-76). For example, in the gene conversion method, a nucleotide sequence corresponding
to the gene is mutagenized in vitro to produce a defective nucleotide sequence, which is
then transformed into the parent *Bacillus* strain to produce a defective gene. By homologous
recombination, the defective nucleotide sequence replaces the endogenous gene. It may be
desirable that the defective nucleotide sequence also comprises a marker for selection of
transformants containing the defective gene.

The *Bacillus* mutant strains may also be constructed by established anti-sense
techniques using a nucleotide sequence complementary to the nucleotide sequence of the
expression of the gene by a *Bacillus* strain may be reduced or inactivated by introducing a
nucleotide sequence complementary to the nucleotide sequence of the gene, which may be
transcribed in the strain and is capable of hybridizing to the mRNA produced in the strain.
Under conditions allowing the complementary anti-sense nucleotide sequence to hybridize
to the mRNA, the amount of protein translated is thus reduced or eliminated.

The *Bacillus* mutant strains may be further constructed by random or specific
mutagenesis using methods well known in the art, including, but not limited to, chemical
mutagenesis (see, for example, Hopwood, *The Isolation of Mutants* in *Methods in
Microbiology* (J.R. Norris and D.W. Ribbons, eds.) pp. 363-433, Academic Press, New York,
1970). Modification of the gene may be performed by subjecting the parent strain to
mutagenesis and screening for mutant strains in which expression of the gene has been
reduced or inactivated. The mutagenesis, which may be specific or random, may be
performed, for example, by use of a suitable physical or chemical mutagenizing agent, use
of a suitable oligonucleotide, or subjecting the DNA sequence to PCR generated
mutagenesis. Furthermore, the mutagenesis may be performed by use of any combination
of these mutagenizing methods.

Examples of a physical or chemical mutagenizing agent suitable for the present
purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), N-methyl-N'-nitrosoguanidine (NTG) O-methyl hydroxylamine, nitrous acid, ethyl methane sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues. When such agents are used, the mutagenesis is typically performed by incubating the parent strain to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions, and selecting for mutants exhibiting reduced or no expression of the gene.

A nucleotide sequence homologous or complementary to a gene described herein may be used from other microbial sources to disrupt the corresponding gene in a Bacillus strain of choice.

In one aspect, the modification of a gene in the Bacillus mutant is unmarked with a selectable marker. Removal of the selectable marker gene may be accomplished by culturing the mutants on a counter-selection medium. Where the selectable marker gene contains repeats flanking its 5' and 3' ends, the repeats will facilitate the looping out of the selectable marker gene by homologous recombination when the mutant strain is submitted to counter-selection. The selectable marker gene may also be removed by homologous recombination by introducing into the mutant strain a nucleic acid fragment comprising 5' and 3' regions of the defective gene, but lacking the selectable marker gene, followed by selecting on the counter-selection medium. By homologous recombination, the defective gene containing the selectable marker gene is replaced with the nucleic acid fragment lacking the selectable marker gene. Other methods known in the art may also be used.

Also described are methods of producing the Bacillus mutant described herein. In one aspect is a method for obtaining a Bacillus mutant described herein, comprising disrupting in a parent Bacillus strain an endogenous mecA gene and an endogenous sinl gene. In another aspect is a method for obtaining a Bacillus mutant described herein, comprising: (a) cultivating a parent Bacillus strain; (a) disrupting an endogenous mecA gene and an endogenous sinl gene in a parent Bacillus strain of (a); and (c) isolating the mutant strain resulting from (b).

Transformed DNA and Related Methods

The Bacillus mutants described herein are useful for producing Bacillus transformants. In one aspect is a method of obtaining a Bacillus transformant, comprising transforming a heterologous polynucleotide into a Bacillus mutant described herein. In another aspect is a method of obtaining a Bacillus transformant, comprising: (a) cultivating a Bacillus mutant described herein; (b) transforming a heterologous polynucleotide into the Bacillus mutant of (a); and (c) isolating the transformant strain resulting from (b).
The transformed DNA described herein can be any DNA of interest. The DNA may be of genomic, cDNA, semisynthetic, synthetic origin, or any combinations thereof. The DNA may be a heterologous polynucleotide that encodes any polypeptide having biological activity of interest or may be a DNA involved in the expression of the polypeptide having biological activity, e.g., a promoter.

The polypeptide having a biological activity may be any polypeptide of interest. The polypeptide may be native or foreign to the *Bacillus* host cell of interest. The polypeptide may be naturally occurring allelic and engineered variations of the below-mentioned polypeptides and hybrid polypeptides.

The term "polypeptide" is not meant herein to refer to a specific length of the encoded product and, therefore, encompasses peptides, oligopeptides, and proteins. The term "polypeptide" also encompasses hybrid polypeptides, which comprise a combination of partial or complete polypeptide sequences obtained from at least two different polypeptides wherein one or more may be foreign to the *Bacillus* cell. Polypeptides further include naturally occurring allelic and engineered variations of a polypeptide.

In one aspect, the polypeptide is an antibody, antigen, antimicrobial peptide, enzyme, growth factor, hormone, immunodilator, neurotransmitter, receptor, reporter protein, structural protein, and transcription factor.

In another aspect, the polypeptide is an oxidoreductase, transferase, hydrolase, lyase, isomerase, or ligase. In a most preferred aspect, the polypeptide is an alpha-glucosidase, aminopeptidase, amylase, carboxydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, glucocerebrosidase, alpha-galactosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phospholipase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, urokinase, or xylanase.

In another aspect, the polypeptide is an albumin, collagen, tropoelastin, elastin, or gelatin.

In another aspect, the polypeptide is a hybrid polypeptide, which comprises a combination of partial or complete polypeptide sequences obtained from at least two different polypeptides wherein one or more may be foreign to the *Bacillus* host cell.

In another aspect, the polypeptide is a fused polypeptide in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide or fragment thereof. A fused polypeptide is produced by fusing a nucleotide sequence (or a portion thereof) encoding one polypeptide to a nucleotide sequence (or a portion thereof) encoding another polypeptide. Techniques for producing fusion polypeptides are known in the art,
and include, ligating the coding sequences encoding the polypeptides so that they are in frame and expression of the fused polypeptide is under control of the same promoter(s) and terminator.

The heterologous polynucleotide encoding a polypeptide of interest may be obtained from any prokaryotic, eukaryotic, or other source. For purposes of the present invention, the term "obtained from" as used herein in connection with a given source shall mean that the polypeptide is produced by the source or by a cell in which a gene from the source has been inserted.

Techniques used to isolate or clone a heterologous polynucleotide encoding a polypeptide of interest are known in the art and include isolation from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of the DNA of interest from such genomic DNA can be effected, e.g., by using the well known polymerase chain reaction (PCR). See, for example, Innis et al., PCR Protocols: A Guide to Methods and Application, Academic Press, New York, 1990. The cloning procedures may involve excision and isolation of a desired nucleic acid fragment comprising the nucleic acid sequence encoding the polypeptide, insertion of the fragment into a vector molecule, and incorporation of the recombinant vector into the Bacillus mutant where multiple copies or clones of the nucleic acid sequence will be replicated. The DNA may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

A heterologous polynucleotide encoding a polypeptide of interest may be manipulated in a variety of ways to provide for expression of the polypeptide in a mutant Bacillus strain. Manipulation of the polynucleotide's sequence prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying polynucleotide sequences utilizing recombinant DNA methods are well known in the art.

A nucleic acid construct comprising a polynucleotide encoding a polypeptide may be operably linked to one or more (e.g., two, several) control sequences capable of directing expression of the coding sequence in a mutant Bacillus strain of the present invention under conditions compatible with the control sequences.

The control sequence may be an appropriate promoter sequence, a nucleotide sequence that is recognized by a mutant Bacillus strain of the present invention for expression of the polynucleotide encoding the polypeptide. The promoter sequence contains transcriptional control sequences that mediate expression of the polypeptide. The promoter may be any nucleotide sequence that shows transcriptional activity in the mutant Bacillus strain, including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either native or foreign to the mutant.
Bacillus strain.

Examples of suitable promoters for directing the transcription of the nucleic acid constructs in a mutant Bacillus strain are the promoters obtained from the Bacillus amyloliquefaciens alpha-amylase gene (amyQ), Bacillus licheniformis alpha-amylase gene (amyL), Bacillus licheniformis penicillinase gene (penP), Bacillus stearothermophilus maltogenic amylase gene (amyM), Bacillus subtilis levansucrase gene (sacB), Bacillus subtilis xylA and xylB genes, E. coli lac operon, E. coli trc promoter (Egon et al., Gene 1988, 69, 301-315), Streptomyces coelicolor agarase gene (dagA), and prokaryotic beta-lactamase gene (Villa-Kamaroff et al., Proc. Natl. Acad. Sci. U.S.A. 1978, 75, 3727-3731), as well as the tac promoter (DeBoer et al., Proc. Natl. Acad. Sci. U.S.A. 1983, 80, 21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in Gilbert et al., Scientific American 1980, 242, 74-94; and in Sambrook et al., 1989, supra.

The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a mutant Bacillus strain to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleotide sequence encoding the heterologous polypeptide. Any terminator that is functional in a Bacillus strain may be used.

The control sequence may also be a suitable leader sequence, a nontranslated region of mRNA that is important for translation by a mutant Bacillus strain. The leader sequence is operably linked to the 5' terminus of the nucleotide sequence encoding the heterologous polypeptide. Any leader sequence that is functional in the mutant Bacillus strain may be used.

The control sequence may also be a signal peptide coding sequence that codes for an amino acid sequence linked to the amino terminus of a polypeptide and directs the encoded polypeptide into the cell's secretory pathway. The 5' end of the coding sequence of the nucleotide sequence may inherently contain a signal peptide coding sequence naturally linked in translation reading frame with the segment of the coding sequence that encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide coding sequence that is foreign to the coding sequence. The foreign signal peptide coding sequence may be required where the coding sequence does not naturally contain a signal peptide coding sequence. Alternatively, the foreign signal peptide coding sequence may simply replace the natural signal peptide coding sequence in order to enhance secretion of the polypeptide. However, any signal peptide coding sequence that directs the expressed polypeptide into the secretory pathway of the mutant Bacillus strain, i.e., secreted into a culture medium, may be used in the present invention.

A recombinant expression vector comprising a nucleotide sequence, a promoter, and transcriptional and translational stop signals may be used for the recombinant production of
a polypeptide of interest. The various nucleic acids and control sequences described herein
may be joined together to produce a recombinant expression vector that may include one or
more (e.g., two, several) convenient restriction sites to allow for insertion or substitution of
the nucleotide sequence encoding the polypeptide at such sites. Alternatively, the nucleotide
sequence may be expressed by inserting the nucleotide sequence or a nucleic acid
construct comprising the sequence into an appropriate vector for expression. In creating the
expression vector, the coding sequence is located in the vector so that the coding sequence
is operably linked with the appropriate control sequences for expression.

The recombinant expression vector may be any vector (e.g., a plasmid or virus) that
can be conveniently subjected to recombinant DNA procedures and can bring about
expression of the nucleotide sequence. The choice of the vector will typically depend on its
compatibility with the mutant Bacillus strain into which the vector is to be introduced. The
vector may be a linear or closed circular plasmid.

The vector may be an autonomously replicating vector, i.e., a vector that exists as an
extrachromosomal entity, the replication of which is independent of chromosomal
replication, e.g., a plasmid, an extrachromosomal element, a minichromosome, or an
artificial chromosome. The vector may contain any means for assuring self-replication.
Alternatively, the vector may be one that, when introduced into the mutant Bacillus strain, is
integrated into the genome and replicated together with the chromosome(s) into which it has
been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids
that together contain the total DNA to be introduced into the genome of the mutant Bacillus
strain, or a transposon, may be used.

The vector may contain one or more (e.g., two, several) selectable markers that
permit easy selection of transformed mutant Bacillus strains. A selectable marker is a gene
the product of which provides for biocide or viral resistance, resistance to heavy metals,
prototrophy to auxotrophs, and the like.

Examples of selectable markers for use in the mutant Bacillus strain include, but are
not limited to, the dal genes from Bacillus subtilis or Bacillus licheniformis, or markers that
confer antibiotic resistance such as ampicillin, chloramphenicol, kanamycin, or tetracycline
resistance. Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3,
TRP1, and URA3.

The vectors may contain one or more (e.g., two, several) elements that permit
integration of the vector into the Bacillus genome or autonomous replication of the vector in
the cell independent of the genome.

For integration into the genome of the mutant Bacillus strain, the vector may rely on
the polynucleotide's sequence encoding the polypeptide of interest or any other element of
the vector for integration into the genome by homologous or nonhomologous recombination. Alternatively, the vector may contain additional nucleotide sequences for directing integration by homologous recombination into the genome of the mutant Bacillus strain at a precise location(s) in the chromosome. To increase the likelihood of integration at a precise location, the integrational elements should preferably contain a sufficient number of nucleic acids, such as 100 to 1,500 base pairs, preferably 400 to 1,500 base pairs, and most preferably 800 to 1,500 base pairs, which have a high degree of identity to the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the mutant Bacillus strain. Furthermore, the integrational elements may be non-encoding or encoding nucleotide sequences. On the other hand, the vector may be integrated into the genome of the mutant Bacillus strain by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the mutant Bacillus strain. The origin of replication may be any plasmid replicator mediating autonomous replication that functions in a cell. The term "origin of replication" or "plasmid replicator" is defined herein as a nucleotide sequence that enables a plasmid or vector to replicate in vivo. Examples of bacterial origins of replication useful in the mutant Bacillus strain are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in E. coli, and pUB1 10, pE194, pTA1060, and pAMβI permitting replication in Bacillus.

The procedures used to ligate the elements described herein to construct the recombinant expression vectors are well known to one skilled in the art (see, e.g., J. Sambrook, E.F. Fritsch, and T. Maniatis, 1989, Molecular Cloning, A Laboratory Manual, 2d edition, Cold Spring Harbor, New York).

The DNA can also be a control sequence, e.g., promoter, for manipulating the expression of a gene of interest. Non-limiting examples of control sequences are described above.

The DNA can further be a nucleic acid construct for inactivating a gene of interest in a Bacillus cell.

The DNA is not to be limited in scope by the specific examples disclosed above, since these examples are intended as illustrations of several aspects of the invention.

Transformation of the DNA into the mutant Bacillus strains can be conducted using techniques known in the art, such as electroporation as described in the Examples section below.

The transformants described herein can be isolated using standard techniques well-
known in the art, including, but not limited to, streak plate isolation, growth in enrichment or selective media, temperature growth selection, filtration, or single cell isolation techniques, such as flow cytometry and microfluidics.

Methods of Producing Polypeptides and Fermentation Products

Polypeptides

As mentioned supra, the *Bacillus* mutants described herein can increase the efficiency in producing *Bacillus* transformants which are useful, e.g., in producing a polypeptide having biological activity. Accordingly, in one aspect is a method of producing a polypeptide having biological activity, comprising: (a) cultivating a *Bacillus* host cell transformed with a heterologous polynucleotide encoding the polypeptide under conditions conducive for production of the polypeptide, wherein the *Bacillus* host cell is a *Bacillus* mutant described herein (e.g., a *Bacillus* mutant comprising a disruption to an endogenous *mecA* gene and an endogenous *sinl* gene); and (b) recovering the polypeptide.

In another aspect is a method of producing a polypeptide, comprising: (a) cultivating a *Bacillus* transformant described herein (e.g., a *Bacillus* mutant comprising a disruption to an endogenous *mecA* gene and an endogenous *sinl* gene); and (b) recovering the polypeptide.

The competent *Bacillus* host cells are cultivated in a nutrient medium suitable for production of a polypeptide of interest using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the polypeptide of interest to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). The secreted substance of interest, e.g., polypeptide or fermentation product, can be recovered directly from the medium.

The polypeptide having biological activity may be detected using methods known in the art that are specific for the substance. These detection methods may include use of specific antibodies, high performance liquid chromatography, capillary chromatography, formation of an enzyme product, disappearance of an enzyme substrate, or SDS-PAGE. For example, an enzyme assay may be used to determine the activity of a polypeptide having enzyme activity. Procedures for determining enzyme activity are known in the art for many enzymes (see, for example, D. Schomburg and M. Salzmann (eds.), *Enzyme*
The resulting polypeptide having biological activity may be isolated by methods known in the art. For example, a polypeptide of interest may be isolated from the cultivation medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation. The isolated polypeptide may then be further purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing (IEF), differential solubility (e.g., ammonium sulfate precipitation), or extraction (see, e.g., Protein Purification, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

Fermentation Products

The *Bacillus* mutants described herein can be used in metabolic engineering, e.g., in the production of a fermentation product. The increased transformation efficiency for the mutants may provide the tools to use *Bacillus* over an existing host, and may permit rapid screening of overexpressed heterologous genes for existing and new metabolic pathways.

"Fermentation" or "fermentation process" refers to any fermentation process or any process comprising a fermentation step. Fermentation processes include fermentation processes used in the consumable alcohol industry (e.g., beer and wine), dairy industry (e.g., fermented dairy products), leather industry, tobacco industry, and specialty or bulk chemical industry.

In one aspect is a method of producing a fermentation product, comprising: (a) cultivating a *Bacillus* transformant described herein (e.g., a *Bacillus* mutant described herein transformed with one or more heterologous polynucleotides that encode one or more polypeptides of a fermentation pathway) under conditions conducive for production of the fermentation product; and (b) recovering the fermentation product.

The *Bacillus* transformant can be any *Bacillus* mutant described herein that is transformed with one or more heterologous fermentation pathway genes, resulting in increased production of a desired fermentation product. Metabolic pathway genes and corresponding engineered transformants for fermentation of a variety of desired fermentation products are known in the art, e.g., the production of isopropanol and n-propanol (WO2012/058603), 3-hydroxypropionic acid (WO2005/118719), malic acid (WO2011/028643), 1,4-butanediol (WO2008/115840), 1,3-butanediol (WO2010/127319), 2-butanol (WO2010/144746), THF (WO2010/141920), caprolactam (WO2010129936), hexamethylenediamine (WO2010129936), levulinic acid (WO2010129936), 2/3-hydroxyisobutyric acid (WO2009/135074), methacrylic acid (WO2009/135074), adipic acid
(WO2009/151728), butadiene (WO201 1/140171), muconate (WO201 1/017560) and 4-hydroxybutanal (WO201 1/047101) (the contents of these applications are hereby incorporated by reference). The Bacillus mutants described herein may provide tools to further improve on producing the fermented products in the references above.

Methods for producing a fermentation product may be performed in a fermentable medium comprising any one or more (e.g., two, several) sugars, such as glucose, fructose, sucrose, cellobiose, xylose, xylulose, arabinose, mannose, galactose, and/or soluble oligosaccharides. In some instances, the fermentation medium is derived from a natural source, such as sugar cane, starch, or cellulose, and may be the result of pretreating the source by enzymatic hydrolysis (saccharification).

In addition to the appropriate carbon sources from one or more (e.g., two, several) sugar(s), the fermentable medium may contain other nutrients or stimulators known to those skilled in the art, such as macronutrients (e.g., nitrogen sources) and micronutrients (e.g., vitamins, mineral salts, and metallic cofactors). In some aspects, the carbon source can be preferentially supplied with at least one nitrogen source, such as yeast extract, N₂₆₇ peptone (e.g., Bacto™ Peptone), or soytone (e.g., Bacto™ Soytone). Nonlimiting examples of vitamins include multivitamins, biotin, pantothenate, nicotinic acid, meso-inositol, thiamine, pyridoxine, para-aminobenzoic acid, folic acid, riboflavin, and Vitamins A, B, C, D, and E. Examples of mineral salts and metallic cofactors include, but are not limited to Na, P, K, Mg, S, Ca, Fe, Zn, Mn, and Cu.

The fermenting microorganism is typically added to the fermentation medium and the fermentation is performed for about 8 to about 96 hours, e.g., about 24 to about 60 hours. The temperature is typically between about 26°C to about 60°C, e.g., about 32°C or 50°C, and about pH 3 to about pH 8, e.g., pH 4-5, 6, or 7.

Cultivation may be performed under anaerobic, substantially anaerobic (microaerobic), or aerobic conditions, as appropriate. Briefly, anaerobic refers to an environment devoid of oxygen, substantially anaerobic (microaerobic) refers to an environment in which the concentration of oxygen is less than air, and aerobic refers to an environment wherein the oxygen concentration is approximately equal to or greater than that of the air. Substantially anaerobic conditions include, for example, a culture, batch fermentation or continuous fermentation such that the dissolved oxygen concentration in the medium remains less than 10% of saturation. Substantially anaerobic conditions also includes growing or resting cells in liquid medium or on solid agar inside a sealed chamber maintained with an atmosphere of less than 1% oxygen. The percent of oxygen can be maintained by, for example, sparging the culture with an N₂/C0₂ mixture or other suitable non-oxygen gas or gases. In some embodiments, the cultivation is performed under
anaerobic conditions or substantially anaerobic conditions.

The methods for producing a fermentation product can employ any suitable fermentation operation mode. For example, batch mode fermentation may be used with a close system where culture media and host microorganism, set at the beginning of fermentation, have no additional input except for the reagents certain reagents, e.g., for pH control, foam control or others required for process sustenance. The process described herein can also be employed in Fed-batch or continuous mode.

The methods for producing a fermentation product may be practiced in several bioreactor configurations, such as stirred tank, bubble column, airlift reactor and others known to those skilled in the art. The methods may be performed in free cell culture or in immobilized cell culture as appropriate. Any material support for immobilized cell culture may be used, such as alginates, fibrous bed, or argyle materials such as chrysotile, montmorillonite KSF and montmorillonite K-10.

A fermentation product can be any substance derived from the fermentation. The fermentation product can be, without limitation, an alcohol (e.g., arabinitol, n-butanol, isobutanol, ethanol, glycerol, methanol, ethylene glycol, 1,3-propanediol [propylene glycol], butanediol, glycerin, sorbitol, and xylitol); an alkane (e.g., pentane, hexane, heptane, octane, nonane, decane, undecane, and dodecane), a cycloalkane (e.g., cyclopentane, cyclohexane, cycloheptane, and cyclooctane), an alkene (e.g. pentene, hexene, heptene, and octene); an amino acid (e.g., aspartic acid, glutamic acid, glycine, lysine, serine, and threonine); a gas (e.g., methane, hydrogen (H₂), carbon dioxide (CO₂), and carbon monoxide (CO)); isoprene; a ketone (e.g., acetone); an organic acid (e.g., acetic acid, acetic acid, adipic acid, ascorbic acid, citric acid, 2,5-diketo-D-gluconic acid, formic acid, fumaric acid, glucaric acid, gluconic acid, glucuronic acid, glutaric acid, 3-hydroxypropionic acid, itaconic acid, lactic acid, malic acid, malonic acid, oxalic acid, oxaloacetic acid, propionic acid, succinic acid, and xylonic acid); and polyketide. The fermentation product can also be protein as a high value product.

In a one aspect, the fermentation product is an alcohol. It will be understood that the term "alcohol" encompasses a substance that contains one or more hydroxyl moieties. The alcohol can be any alcohol, including, but not limited to propanol, n-butanol, iso-butanol, isobutanol, ethanol, methanol, arabinitol, butanediol, ethylene glycol, glycerin, glycerol, 1,3-propanediol, sorbitol, or xylitol. See, for example, Gong, et al., 1999. Ethanol production from renewable resources, in Advances in Biochemical Engineering/Biotechnology, Scheper, T., ed., Springer-Verlag Berlin Heidelberg, Germany, 65: 207-241; Silveira, M. M., and Jonas, R., Appl. Microbiol. Biotechnol. 2002, 59, 400-408; Nigam, P., and Singh, D., Process Biochemistry 1995, 30, 117-124; Ezeji, et al., World Journal of Microbiology and
Biotechnology 2003, 19, 595-603.

In one aspect, the fermentation product is propanol, such as isopropanol and/or n-propanol (see WO2012/058603, the content of which is hereby incorporated by reference).

In another aspect, the fermentation product is an alkane. The alkane can be any unbranched or a branched alkane, including, but not limited to pentane, hexane, heptanes, octane, nonane, decane, undecane, or dodecane.

In another aspect, the fermentation product is a cycloalkane, e.g., cyclopentane, cyclohexane, cycloheptane, or cyclooctane.

In another aspect, the fermentation product is an alkene. The alkene can be any unbranched or a branched alkene, including, but not limited to pentene, hexane, heptene, or octene.

In another aspect, the fermentation product is an amino acid. The amino acid can be any amino acid, including, but not limited to aspartic acid, glutamic acid, glycine, lysine, serine, or threonine. See, for example, Richard, A., and Margaritis, A., Biotechnol. Bioeng. 2004, 87, 501-515.

In another preferred aspect, the fermentation product is a gas. The gas can be any gas, including, but not limited to methane, H₂, CO₂, or CO. See, for example, Kataoka, et al., Water Science and Technology 1997, 36, 41-47; and Gunaseelan V. N., Biomass and Bioenergy, 1997, 13, 83-1 14.

In another aspect, the fermentation product is isoprene.

In another aspect, the fermentation product is a ketone. It will be understood that the term "ketone" encompasses a substance that contains one or more ketone moieties. In one aspect, the ketone is acetone.

In another aspect, the fermentation product is an organic acid. The organic acid can be any organic acid, including, but not limited to acetic acid, acetic acid, adipic acid, ascorbic acid, citric acid, 2,5-diketo-D-gluconic acid, formic acid, fumaric acid, glucaric acid, gluconic acid, glucuronic acid, glutaric acid, 3-hydroxypropionic acid, itaconic acid, lactic acid, malic acid, malonic acid, oxalic acid, propionic acid, succinic acid, or xylonic acid. See, for example, Chen, R., and Lee, Y. Y., Appl. Biochem. Biotechnol. 1997, 63-65, 435-448. In some aspects, the fermentation product is an amino acid. The amino acid can be any amino acid, including, but not limited to aspartic acid, glutamic acid, glycine, lysine, serine, or threonine. See, for example, Richard, A., and Margaritis, A., Biotechnol. Bioeng. 2004, 87, 501-515.

In another aspect, the fermentation product is polyketide.

Suitable assays to test for the production of the fermentation product can be performed using methods known in the art, as described above for polypeptides. For
example, the fermentation product (and other organic compounds, such as side products) can be analyzed by methods such as HPLC (High Performance Liquid Chromatography), GC-MS (Gas Chromatography Mass Spectroscopy) and LC-MS (Liquid Chromatography-Mass Spectroscopy) or other suitable analytical methods using routine procedures well known in the art. The release of the fermentation product in the fermentation broth can also be tested with the culture supernatant. Byproducts and residual sugar in the fermentation medium (e.g., glucose) can be quantified by HPLC using, for example, a refractive index detector for glucose and alcohols, and a UV detector for organic acids (Lin et al., *Biotechnol Bioeng* 2005, 90, 775-779), or using other suitable assay and detection methods well known in the art.

Recovery of the fermentation product from the fermentation medium can be conducted using any procedure known in the art including, but not limited to, chromatography (e.g., size exclusion chromatography, adsorption chromatography, ion exchange chromatography), electrophoretic procedures, differential solubility, distillation, extraction (e.g., liquid-liquid extraction), pervaporation, extractive filtration, membrane filtration, membrane separation, reverse osmosis, ultrafiltration, or crystallization.

The following examples are provided by way of illustration and are not intended to be limiting of the invention.
Examples

Chemicals used as buffers and substrates were commercial products of at least reagent grade.

Strains

*Escherichia coli*

One Shot™ TOP10 chemically competent *E. coli* cells (Invitrogen, Carlsbad, CA) and Sure™ Competent cells (Stratagene, La Jolla, CA) were used for routine plasmid constructions and propagation.

*B. licheniformis*

*B. licheniformis* SJ1904 (U.S. Patent No. 5,733,753) was used as a host for *mecA* and *sinl* disruption.

Media

*Bacillus* strains were grown on TBAB (Tryptose Blood Agar Base, Difco Laboratories, Sparks, MD, USA) or LB agar (10 g/l Tryptone, 5 g/l yeast extract, 5 g/l NaCl, 15 g/l agar) plates or in LB liquid medium (10 g/l Tryptone, 5 g/l yeast extract, 5 g/l NaCl).

To select for erythromycin resistance, agar media were supplemented with 1 µg/ml erythromycin + 25 µg/ml lincomycin and liquid media were supplemented with 5 µg/ml erythromycin. To select for spectinomycin resistance, agar media were supplemented with 120 µg/ml spectinomycin.

Spizizen I medium consists of 1 x Spizizen salts (6 g/l KH₂PO₄, 14 g/l K₂HPO₄, 2 g/l (NH₄)₂SO₄, 1 g/l sodium citrate, 0.2 g/l MgSO₄·pH 7.0), 0.5% glucose, 0.1% yeast extract, and 0.02% casein hydrolysate.

Spizizen II medium consists of Spizizen I medium supplemented with 0.5 mM CaCl₂, and 2.5 mM MgCl₂.

Example 1: Construction of a *B. licheniformis* mec^-disrupted strain (TaHy9).

Plasmid pBM294 was designed to delete 500 bp within the *B. licheniformis mecA* gene. Genomic DNA was isolated from *B. licheniformis* SJ1904 according the method previously described (Pitcher et. al, *Lett. Appl. Microbiol.*, 1989, 8, 151-156). A 323 bp fragment of the *B. licheniformis* SJ1904 chromosome, including the first 67 bp of the *mecA* coding sequence, was amplified by PCR using primers 0612056 and 0612057 shown below.

Primer 0612056 (SEQ ID NO: 5):

5'-GAATTCCATTAATAGCTGCTG-3'

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Primer 0612057 (SEQ ID NO: 6):
5'-TCCATACTCTTCAGCATGCTTTGATATACCCGT-3'

A cleavage site for restriction enzyme EcoRI (bold) was incorporated into primer 0612056. Primer 0612057 incorporates 18 bp (underlined) corresponding to bp 568 to 588 of the mecA coding sequence.

A second 288 bp fragment of the *B. licheniformis* SJ1904 chromosome, including the segment from nucleotides 568 to 639 of the mecA coding sequence, was amplified by PCR using primers 0612058 and 0612060 shown below.

Primer 0612058 (SEQ ID NO: 7):
5'-ACGGTGATATCAGACCATGTGCTGAAAGAGTATGGA-3'

Primer 0612060 (SEQ ID NO: 8):
5'-CTCGAGCGCATCCTCCAAAAATC-3'

A cleavage site for the XhoI restriction enzyme (bold) was incorporated into primer 0612060. Primer 0612058 incorporates 18 bp (underlined) corresponding to bp 47 to 67 of the mecA coding sequence. Primers 0612057 and 0612058 are complementary.

The respective DNA fragments were amplified by PCR using the Expand High Fidelity™ PCR system (Roche Diagnostics, Mannheim, Germany). The PCR amplification reaction mixture contained 4 µl (-1 µg) of *B. licheniformis* SJ1904 genomic DNA, 1 µl of sense primer (50 pmol/µl), 1 µl of anti-sense primer (50 pmol/µl), 10 µl of 5X PCR buffer with 15 mM MgCl₂, 1 µl of dNTP mix (10 mM each), 32.25 µl water, and 0.75 µl (3.5 U/µl) DNA polymerase mix. An Eppendorf Mastercycler thermocycler was used to amplify the fragment with the following settings: One cycle at 94°C for 2 minutes; 10 cycles each at 94°C for 15 seconds, 58°C for 30 seconds, 72°C for 20 seconds; 15 cycles each at 94°C for 15 seconds, 58°C for 30 seconds, 72°C for 20 seconds plus 5 second elongation at each successive cycle, one cycle at 72°C for 7 minutes; and 4°C hold. The PCR products were purified from a 1.2% agarose (Amresco, Solon, OH) gel with 1x TBE buffer using the Qiagen QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions.

The purified PCR products were used in a subsequent PCR reaction to create a single fragment using splice overlapping PCR (SOE) using the Expand High Fidelity™ PCR system (Roche Diagnostics) as follows. The PCR amplification reaction mixture contained 2 µl (-50 ng) of gel purified PCR product from primer combination 0612056/0612057, 2 µl (-50 ng) of gel purified PCR product from primer combination 0612058/0612060, 1 µl of primer 0612056 (50 pmol/µl), 1 µl of primer 0612060 (50 pmol/µl), 10 µl of 5X PCR buffer with 15 mM MgCl₂, 1 µl of dNTP mix (10 mM each), 32.25 µl water, and 0.75 µl (3.5 U/µl)
DNA polymerase mix. An Eppendorf Mastercycler thermocycler was used to amplify the fragment with the following settings: One cycle at 94°C for 2 minutes; 10 cycles each at 94°C for 15 seconds, 58°C for 30 seconds, 72°C for 40 seconds; 15 cycles each at 94°C for 15 seconds, 58°C for 30 seconds, 72°C for 40 seconds plus 5 second elongation at each successive cycle, one cycle at 72°C for 7 minutes; and 4°C hold. The resulting 611 bp PCR product was purified from a 1.2% agarose (Amresco) gel with 1x TBE buffer using the Qiagen QIAquick Gel Extraction Kit (Qiagen, Inc.) according to manufacturer's instructions.

The purified PCR product was cloned into plasmid pCR2.1-TOPO (Invitrogen) according to manufacturer's instructions, resulting in a plasmid designated pBM293. Plasmid pBM293 and plasmid pNNB194 (U.S. Patent No. 5,958,728) were digested with restriction enzymes Xho\ and EcoRI to isolate the 606 bp insert fragment and vector fragment, respectively. These fragments were isolated by 1% agarose gel electrophoresis using TBE buffer followed by purification using the Qiagen QIAquick Gel Extraction Kit (Qiagen, Inc.) according to manufacturer's instructions. The fragments were ligated using a Rapid DNA Ligation Kit following the manufacturer's instructions. A 2 µl aliquot of the ligation was used to transform E. coli Sure™ cells according to the manufacturer's instructions. Plasmid DNA was prepared from E. coli transformants and digested using restriction enzymes EcoRI and Xho\, followed by 0.7% agarose gel electrophoresis using TBE buffer and the plasmid identified as having the correct restriction pattern was designated pBM294.

The temperature-sensitive plasmid pBM294 was incorporated into the genome of B. licheniformis SJ1904 by chromosomal integration and excision according to the method previously described (U.S. Patent No. 5,843,720). B. licheniformis SJ1904 transformants containing plasmid pBM294 were grown on TBAB selective medium at 50°C to force integration of the vector. Desired integrants were chosen based on their ability to grow on TBAB erythromycin/lincomycin selective medium at 50°C. Integrants were then grown without selection in LB medium at 37°C to allow excision of the integrated plasmid. Cells were plated on LB plates and screened for erythromycin-sensitivity.

Genomic DNA was prepared from several erythromycin/lincomycin sensitive isolates above accordingly to the method previously described (Pitcher et. al, supra). Genomic PCR confirmed disruption of mecA and the resulting strain was designated TaHY9.

Example 2: Transformation efficiency of a B. licheniformis meaA-disrupted strain (TaHy9).

The B. licheniformis mec\-disrupted strain TaHy9 from Example 1 was spread onto LB agar plates to obtain confluent growth after incubation at 37°C overnight. After overnight
incubation, approximately 2-3 ml of Spizizen I medium was added to each plate. Cells were scraped using sterile spreaders and transferred into 15 ml Falcon 2059 tubes. Approximately 500 μl of this culture was used to inoculate 50 ml Spizizen I medium containing 1% xylose as the sole carbon source. Growth was monitored using a Klett densitometer. At each cell density corresponding to Klett unit 140, 160, 180, and 200, 250 μl of the culture plus 250 μl Spizizen II medium containing 2 mM EGTA was added to a Falcon 2059 tube. One microgram of transforming DNA (B. licheniformis MDT232 chromosomal DNA containing a spectinomycin resistance expression cassette integrated at the glpD locus; see WO2008/079895) was added to each tube. Two microliters of 50 μg/ml spectinomycin was also included in the transformation mix. Tubes were incubated at 37°C on a rotational shaker set at 250 rpm for 1 hour. Transformation reactions were plated to LB agar plates containing 120 μg/ml of spectinomycin. Colonies were counted the following day to determine transformation efficiency.

A B. licheniformis competent state in a mecA disrupted strain was reached during the exponential growth phase and declined as cells entered stationary phase (Figure 7). The highest transformation efficiency was obtained at a Klett densitometer reading of 160 and transformation efficiency declined as cells reached stationary phase.

Example 3: DNA microarray analysis of a B. licheniformis meaA-disrupted strain (TaHy9).

In order to obtain additional understanding of B. licheniformis competence development, DNA microarray technology was used to compare global transcription profiles in the B. licheniformis strains TaHy9 (supra) and MMar2 (B. licheniformis SJ1904 amyLv.Xylp-comK, a B. licheniformis strain containing a second copy of the comK gene under transcriptional control of the xylose inducible promoter; see US2010/0028944). Strains TaHy9 and MMar2 were grown in triplicate shake flask cultures containing Spizizen I + 1% xylose medium to 160 reading on a Klett densitometer as described in Example 2. RNA samples were purified from triplicate shake flask cultures and DNA microarray analysis was conducted using custom Affymetrix microarray chips (Affymetrix Inc., Santa Clara, CA) designed for use with B. subtilis strains 168, A164 and B. licheniformis SJ1904. RNA was isolated using the FastRNA™ Pro Blue Kit (MP Biomedicals, LLC, Solon, Ohio), according to manufacturer’s recommendations for bacterial RNA isolation. Cells were disrupted two times for 40 seconds at maximum speed (speed 6) in the FastPrep instrument. In addition, 45 μg of RNA from each sample was further purified using in an RNeasy™ column (Qiagen, Inc.) according to manufacturer’s specifications. The quality, integrity and concentrations of the RNA samples were measured using a Bioanalyzer instrument (Agilent Technologies...
Inc., Santa Clara, CA). RNA samples and custom Affymetrix microarray chips were submitted to the UCLA Clinical Microarray Core Research Facility (Los Angeles, California, USA) for labeling, hybridization and scanning. Microarray data were analyzed using a custom script as previously described (Gillespie et al., *BMC Research Notes* 2010, 3(81)). Data were normalized using Robust Multi-array Average (RMA) (Irizarry et al., *Biostatistics* 2003, 4, 249-264) followed by differential expression analysis using Linear Models for Microarray Data (Limma) software (Smyth. 2005. Limma: Linear models for microarray data. In: *Bioinformatics and Computational Biology Solutions using R and Bioconductor*, Springer, New York, pp. 397-420).

The microarray data revealed significantly increased transcript levels for *epsH*, *tapA*, *sigW* and *tasA* genes in *Bacillus licheniformis* strain TaHy9 compared to strain MMar2 (*p* < 0.05).

**Example 4: Construction of a *B. licheniformis* meaA-disrupted + s/n/-disrupted strain (BaC0155).**

In light of the microarray data of Example 3, a *B. licheniformis* meca sinl double mutant was constructed to test for improved transformation efficiency.

A plasmid designated pBM317, containing an oriT DNA sequence, was constructed to serve as an *E. coli/Bacillus* shuttle vector when using conjugation to introduce plasmid DNA from a *Bacillus* host strain to a *Bacillus* recipient strain. The oriT DNA segment was PCR amplified from plasmid pSHV002 (US 5,891,701) using primers 1200090 and 1200091 below.

Primer 1200090 (SEQ ID NO: 9):

5'-GATAGCTTGGA GTTCTAGAG CGGCCGCATT ATTAATCTGT TCAGC-3'

Primer 1200091 (SEQ ID NO: 10):

5'-GAGCTCCACC GCGTGGCGCG CCGCTGCCCT TTATGCCGAG TG-3'

The 608 bp oriT DNA fragment was PCR amplified using the Expand High Fidelity™ PCR system (Roche Diagnostics) with a reaction mixture containing 1 µl (0.1 13 ng/µl) pSHV002 plasmid DNA, 1 µl primer 1200090 (50 pmol/µl), 1 µl primer 1200091 (50 pmol/µl), 10 µl of 5X PCR buffer with 15 mM MgCl₂, 1 µl of dNTP mix (10 mM each), 35.25 µl water, and 0.75 µl (3.5 U/µl) DNA polymerase mix. An Eppendorf Mastercycler thermocycler was used to amplify the fragment with the following settings: One cycle at 94°C for 2 minutes; 10 cycles each at 94°C for 15 seconds, 58°C for 30 seconds, 72°C for 30 seconds; 15 cycles each at 94°C for 15 seconds, 58°C for 30 seconds, 72°C for 30 seconds plus 5 second elongation at each successive cycle, one cycle at 72°C for 7 minutes; and 4°C hold. The
resulting PCR product was purified using the Qiagen QIAquick PCR Purification Kit (Qiagen, Inc.) according to the manufacturer's instructions.

The 608 bp PCR amplified oriT fragment was cloned into plasmid pNNB194 (U.S. Patent No. 5,958,728) which had been previously digested with restriction enzyme NotI using Clontech In-Fusion HD Cloning System (Clontech laboratories, Inc., Mountain View, CA) according to the manufacturer's instructions. A 2 µl aliquot of the In-fusion mix was used to transform E. coli Stellar™ cells according to the manufacturer's instructions. Plasmid DNA was prepared from E. coli transformants and digested using restriction enzyme A/oil, followed by 1.0% agarose gel electrophoresis using TBE buffer. The plasmid identified as having the correct restriction pattern was designated pBM317.

Plasmid pBM319 was designed to delete the entire 177 bp sinl open reading frame. Genomic DNA was isolated from B. licheniformis SJ1904 according to the method previously described (Pitcher et al, supra). A 434 bp fragment of the B. licheniformis SJ1904 chromosome, flanking the sinl coding sequence at the 5' end, was amplified by PCR from B. licheniformis SJ1904 genomic DNA using primers 1200749 and 1200750 shown below.

Primer 1200749 (SEQ ID NO: 11):

5'-GAATTCGCCAGAAGCGACCGC-3'

Primer 1200750 (SEQ ID NO: 12):

5'-CCTTCCCTTC GATATTATAG CACATACATT TCCCCCCCAA AATACTTGAT-3'

A cleavage site for restriction enzyme EcoRI (bold) was incorporated into primer 1200749.

A second 430 bp fragment of the B. licheniformis SJ1904 chromosome, flanking the sinl coding sequence at the 3' end was amplified by PCR from B. licheniformis SJ1904 genomic DNA using primers 1200751 and 1200752 shown below.

Primer 1200751 (SEQ ID NO: 13):

5'-ATCAAGTATT TTGGGGGGGA AATGTATGTG CTATAATTC GAAAGGAAGG-3'

Primer 1200752 (SEQ ID NO: 14):

5'-CTCGAGAAATGCCAAGGGAG-3'

A cleavage site for restriction enzyme XhoI was incorporated into primer 1200752. Primers 1200750 and 1200751 are complementary.

The respective gene fragments were amplified by PCR using the Expand High Fidelity™ PCR system (Roche Diagnostics) with a reaction mixture containing 4 µl (1 µg) of B. licheniformis SJ1904 genomic DNA, 1 µl of sense primer (50 pmol/µl), 1 µl of anti-sense primer (50 pmol/µl), 10 µl of 5X PCR buffer with 15 mM MgCl₂, 1 µl of dNTP mix (10
mM each), 32.25 μl water, and 0.75 μl (3.5 U/μl) DNA polymerase mix. An Eppendorf Mastercycler thermocycler was used to amplify the fragment with the following settings: One cycle at 94°C for 2 minutes; 10 cycles each at 94°C for 15 seconds, 58°C for 30 seconds, 72°C for 20 seconds; 15 cycles each at 94°C for 15 seconds, 58°C for 30 seconds, 72°C for 20 seconds plus 5 second elongation at each successive cycle, one cycle at 72°C for 7 minutes; and 4°C hold. The PCR products were purified from a 1.0% agarose (Amresco) gel with 1x TBE buffer using the Qiagen QIAquick Gel Extraction Kit (Qiagen, Inc.) according to the manufacturer's instructions.

The purified PCR products were used in a second PCR reaction to create a single fragment using splice overlapping PCR (SOE) using the Expand High Fidelity™ PCR system (Roche Diagnostics) as follows. The PCR amplification reaction mixture contained 2 μl of gel purified PCR product from primer combination 1200749/1200750, 2 μl of gel purified PCR product from primer combination 1200751/1200752, 1 μl of primer 0612056 (50 pmol/μl), 1 μl of primer 0612060 (50 pmol/μl), 10 μl of 5X PCR buffer with 15 mM MgCl₂, 1 μl of dNTP mix (10 mM each), 32.25 μl water, and 0.75 μl (3.5 U/μl) DNA polymerase mix. An Eppendorf Mastercycler thermocycler was used to amplify the fragment with the following settings: One cycle at 94°C for 2 minutes; 10 cycles each at 94°C for 15 seconds, 58°C for 30 seconds, 72°C for 40 seconds; 15 cycles each at 94°C for 15 seconds, 58°C for 30 seconds, 72°C for 40 seconds plus 5 second elongation at each successive cycle, one cycle at 72°C for 7 minutes; and 4°C hold. The resulting 814 bp PCR product was purified from a 1.0% agarose (Amresco) gel with 1x TBE buffer using the Qiagen QIAquick Gel Extraction Kit (Qiagen, Inc.) according to the manufacturer's instructions.

The purified PCR product was cloned into plasmid pCR2.1-TOPO (Invitrogen) according to the manufacturer's instructions. The resulting plasmid and plasmid pBM317 were digested with restriction enzymes XhoI and EcoRI to isolate the 808 bp insert fragment and vector fragment, respectively. These fragments were isolated by 1% agarose gel electrophoresis using TBE buffer followed by purification using the Qiagen QIAquick Gel Extraction Kit (Qiagen, Inc.) according to manufacturer's instructions. The fragments were ligated using a Rapid DNA Ligation Kit following the manufacturer's instructions. A 2 μl aliquot of the ligation was used to transform E. coli Stellar™ cells according to the manufacturer's instructions. Plasmid DNA was prepared from E. coli transformants and digested using restriction enzymes EcoRI and XhoI, followed by 0.7% agarose gel electrophoresis using TBE buffer. The plasmid identified as having the correct restriction pattern was designated pBM319.

Plasmid pBM319 was incorporated into the genome of B. licheniformis SJ1904 by chromosomal integration and excision of the temperature-sensitive plasmid pBM319. B.
licheniformis SJ1904 transformants containing plasmid pBM294 were grown on TBAB selective medium at 50°C to force integration of the vector. Desired integrants were chosen based on their ability to grow on TBAB erythromycin/lincomycin selective medium at 50°C. Integrants were then grown without selection in LB medium at 37°C to allow excision of the integrated plasmid. Cells were plated on LB plates and screened for erythromycin-sensitivity. Genomic DNA was prepared from several erythromycin/lincomycin sensitive isolates according to the method previously described (Pitcher et al., supra). Genomic PCR confirmed disruption of the mecA gene. The resulting strain was designated BaC0155.

Example 5: Transformation efficiency of a B. licheniformis mec^-disrupted + sinl-disrupted strain (BaC0155).

B. licheniformis strains TaHy9 and BaC0155 were grown in Spizizen I medium and transformed with genomic DNA (B. licheniformis MDT232 chromosomal DNA containing a spectinomycin resistance expression cassette integrated at the glpD locus; see WO2008/079895), as described supra. The results of two independent experiments are shown in Figure 8. Based on these experiments, disruption of both the sinl gene and the mecA gene (strain BaC0155) roughly doubles the transformation efficiency when compared to disruption of the mecA gene alone (strain TaHy9).

Although the foregoing has been described in some detail by way of illustration and example for the purposes of clarity of understanding, it is apparent to those skilled in the art that any equivalent aspect or modification may be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention.

In some aspects, the invention may be described by the following numbered paragraphs:

[1] An isolated mutant Bacillus strain, comprising disruption to an endogenous mecA gene and disruption to an endogenous sinl gene.

[2] The isolated mutant of paragraph [1], wherein the endogenous mecA gene (a) encodes for a polypeptide having at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 2 or 16; (b) comprises a coding sequence that hybridizes under at least low, medium, medium-high, high, or very high stringency conditions with the full-length complementary strand of SEQ ID NO: 1 or 15; or (c) comprises a coding sequence that has at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%,
at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 1 or 15.

[3] The isolated mutant of paragraph [1] or [2], wherein the endogenous mecA gene encodes a polypeptide having at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 2 or 16.

[4] The isolated mutant of any one of paragraphs [1]-[3], wherein the endogenous mecA gene encodes a polypeptide comprising or consisting of SEQ ID NO: 2 or 16.

[5] The isolated mutant of any one of paragraphs [1]-[4], wherein the endogenous mecA gene comprises a coding sequence that hybridizes under at least low, medium, medium-high, high, or very high stringency conditions with the full-length complementary strand of SEQ ID NO: 1 or 15.

[6] The isolated mutant of any one of paragraphs [1]-[5], wherein the endogenous mecA gene comprises a coding sequence that has at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 1 or 15.

[7] The isolated mutant of any one of paragraphs [1]-[6], wherein the coding sequence of the endogenous mecA gene comprises or consists of SEQ ID NO: 1 or 15.

[8] The isolated mutant of any one of paragraphs [1]-[7], wherein disruption to the endogenous mecA gene occurs in the coding sequence and/or promoter sequence.

[9] The isolated mutant of any one of paragraphs [1]-[8], wherein the endogenous sinl gene (a) encodes for a polypeptide having at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 4 or 18; (b) comprises a coding sequence that hybridizes under at least low, medium, medium-high, high, or very high stringency conditions with the full-length complementary strand of SEQ ID NO: 3 or 17; or (c) comprises a coding sequence that has at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 3 or 17.
[10] The isolated mutant of any one of paragraphs [1]-[9], wherein the endogenous sinl gene encodes a polypeptide having at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 4 or 18.

[11] The isolated mutant of any one of paragraphs [1]-[10], wherein the endogenous sinl gene encodes a polypeptide comprising or consisting of SEQ ID NO: 4 or 18.

[12] The isolated mutant of any one of paragraphs [1]-[11], wherein the endogenous sinl gene comprises a coding sequence that hybridizes under at least low, medium, medium-high, high, or very high stringency conditions with the full-length complementary strand of SEQ ID NO: 3 or 17.

[13] The isolated mutant of any one of paragraphs [1]-[12], wherein the endogenous sinl gene comprises a coding sequence that has at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 3 or 17.

[14] The isolated mutant of any one of paragraphs [1]-[13], wherein the coding sequence of the endogenous sinl gene comprises or consists of SEQ ID NO: 3 or 17.

[15] The isolated mutant of any one of paragraphs [1]-[14], wherein disruption to the endogenous sinl gene occurs in the coding sequence and/or promoter sequence.

[16] The isolated mutant of any one of paragraphs [1]-[15], wherein the mutant produces at least 25% less (e.g., at least 50% less, at least 60% less, at least 70% less, at least 80% less, or at least 90% less) of the polypeptide encoded by the endogenous mecA gene compared to the parent Bacillus strain that lacks disruption to the endogenous mecA gene and lacks disruption to the endogenous sinl gene, when cultivated under identical conditions.

[17] The isolated mutant of any one of paragraphs [1]-[16], wherein the endogenous mecA gene is inactivated.

[18] The isolated mutant of any one of paragraphs [1]-[17], wherein the mutant produces at least 25% less (e.g., at least 50% less, at least 60% less, at least 70% less, at least 80% less, or at least 90% less) of the polypeptide encoded by the endogenous sinl gene
compared to the parent Bacillus strain that lacks disruption to the endogenous mecA gene and lacks disruption to the endogenous sinl gene, when cultivated under identical conditions.

[19] The isolated mutant of any one of paragraphs [1]-[18], wherein the endogenous sinl gene is inactivated.

[20] The isolated mutant of any one of paragraphs [1]-[19], wherein the mutant has improved transformation efficiency compared to the parent Bacillus strain that lacks disruption to the endogenous mecA gene and lacks disruption to the endogenous sinl gene, when cultivated under identical conditions.

[21] The isolated mutant of any one of paragraphs [1]-[20], wherein the mutant is capable of producing at least 10-fold (e.g., at least 100-fold, at least 1000-fold, at least 10000-fold, or at least 100000-fold) more transformants compared to the parent Bacillus strain that lacks disruption to the endogenous mecA gene and lacks disruption to the endogenous sinl gene, when cultivated under identical conditions.

[22] The isolated mutant of any one of paragraphs [1]-[21], wherein the Bacillus strain is selected from Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus brevis, Bacillus circulans, Bacillus clausii, Bacillus coagulans, Bacillus firmus, Bacillus lautus, Bacillus lentus, Bacillus licheniformis, Bacillus megaterium, Bacillus pumilus, Bacillus stearothermophilus, Bacillus subtilis, or Bacillus thuringiensis.

[23] The isolated mutant of paragraph [22], wherein the Bacillus strain is a Bacillus licheniformis strain.

[24] The isolated mutant of paragraph [22], wherein the Bacillus strain is a Bacillus subtilis strain.


[26] A method for obtaining the isolated mutant of any one of paragraphs [1]-[24], comprising:

(a) cultivating a parent Bacillus strain;
(b) disrupting an endogenous mecA gene and an endogenous sinl gene in the parent Bacillus strain of (a); and
(c) isolating the mutant strain resulting from (b).

[27] A method for obtaining an isolated Bacillus transformant, comprising transforming a heterologous polynucleotide into the isolated Bacillus mutant of any one of paragraphs [1]-[24].

[28] A method for obtaining an isolated Bacillus transformant, comprising:

(a) cultivating the isolated Bacillus mutant of any one of paragraphs [1]-[24];
(b) transforming a heterologous polynucleotide into the Bacillus mutant of (a); and
(c) isolating the transformant strain resulting from (b).

[29] An isolated Bacillus transformant produced by the method of paragraph [27] or [28].

[30] A method for obtaining a Bacillus transformant, comprising transforming a heterologous polynucleotide into a Bacillus mutant, wherein the mutant comprises disruption to an endogenous mecA gene and disruption to an endogenous sinl gene.

[31] The method of paragraph [30], wherein the endogenous mecA gene (a) encodes for a polypeptide having at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 2 or 16; (b) comprises a coding sequence that hybridizes under at least low, medium, medium-high, high, or very high stringency conditions with the full-length complementary strand of SEQ ID NO: 1 or 15; or (c) comprises a coding sequence that has at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 1 or 15.

[32] The method of paragraph [30] or [31], wherein the endogenous mecA gene encodes a polypeptide having at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 2 or 16.

[33] The method of any one of paragraphs [30]-[32], wherein the endogenous mecA gene encodes a polypeptide comprising or consisting of SEQ ID NO: 2 or 16.
[34] The method of any one of paragraphs [30]-[33], wherein the endogenous mecA gene comprises a coding sequence that hybridizes under at least low, medium, medium-high, high, or very high stringency conditions with the full-length complementary strand of SEQ ID NO: 1 or 15.

[35] The method of any one of paragraphs [30]-[34], wherein the endogenous mecA gene comprises a coding sequence that has at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 1 or 15.

[36] The method of any one of paragraphs [30]-[35], wherein the coding sequence of the endogenous mecA gene comprises or consists of SEQ ID NO: 1 or 15.

[37] The method of any one of paragraphs [30]-[36], wherein disruption to the endogenous mecA gene occurs in the coding sequence and/or promoter sequence.

[38] The method of any one of paragraphs [30]-[37], wherein the endogenous sinl gene (a) encodes for a polypeptide having at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 4 or 18; (b) comprises a coding sequence that hybridizes under at least low, medium, medium-high, high, or very high stringency conditions with the full-length complementary strand of SEQ ID NO: 3 or 17; or (c) comprises a coding sequence that has at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 3 or 17.

[39] The method of any one of paragraphs [30]-[38], wherein the endogenous sinl gene encodes a polypeptide having at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 4 or 18.

[40] The method of any one of paragraphs [30]-[39], wherein the endogenous sinl gene encodes a polypeptide comprising or consisting of SEQ ID NO: 4 or 18.

[41] The method of any one of paragraphs [30]-[40], wherein the endogenous sinl gene comprises a coding sequence that hybridizes under at least low, medium, medium-high,
high, or very high stringency conditions with the full-length complementary strand of SEQ ID NO: 3 or 17.

[42] The method of any one of paragraphs [30]-[41], wherein the endogenous sinl gene comprises a coding sequence that has at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 3 or 17.

[43] The method of any one of paragraphs [30]-[42], wherein the coding sequence of the endogenous sinl gene comprises or consists of SEQ ID NO: 3 or 17.

[44] The method of any one of paragraphs [30]-[43], wherein disruption to the endogenous sinl gene occurs in the coding sequence and/or promoter sequence.

[45] The method of any one of paragraphs [30]-[44], wherein the mutant produces at least 25% less (e.g., at least 50% less, at least 60% less, at least 70% less, at least 80% less, or at least 90% less) of the polypeptide encoded by the endogenous mecA gene compared to the parent Bacillus strain that lacks disruption to the endogenous mecA gene and lacks disruption to the endogenous sinl gene, when cultivated under identical conditions.

[46] The method of any one of paragraphs [30]-[45], wherein the endogenous mecA gene is inactivated.

[47] The method of any one of paragraphs [30]-[46], wherein the mutant produces at least 25% less (e.g., at least 50% less, at least 60% less, at least 70% less, at least 80% less, or at least 90% less) of the polypeptide encoded by the endogenous sinl gene compared to the parent Bacillus strain that lacks disruption to the endogenous mecA gene and lacks disruption to the endogenous sinl gene, when cultivated under identical conditions.

[48] The method of any one of paragraphs [30]-[47], wherein the endogenous sinl gene is inactivated.

[49] The method of any one of paragraphs [30]-[48], wherein the mutant has improved transformation efficiency compared to the parent Bacillus strain that lacks disruption to the endogenous mecA gene and lacks disruption to the endogenous sinl gene, when cultivated under identical conditions.
[50] The method of any one of paragraphs [30]-[49], wherein the mutant is capable of producing at least 10-fold (e.g., at least 100-fold, at least 1000-fold, at least 10000-fold, or at least 100000-fold) more transformants compared to the parent *Bacillus* strain that lacks disruption to the endogenous *mecA* gene and lacks disruption to the endogenous *sinI* gene, when cultivated under identical conditions.

[51] The method of any one of paragraphs [30]-[50], wherein the *Bacillus* strain is selected from *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus firmus*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, or *Bacillus thuringiensis*.

[52] The method of paragraph [51], wherein the *Bacillus* strain is a *Bacillus licheniformis* strain.

[53] The method of paragraph [51], wherein the *Bacillus* strain is a *Bacillus subtilis* strain.

[54] The method of any one of paragraphs [30]-[54] further comprising isolating the *Bacillus* transformant.

[55] A method of producing a polypeptide, comprising:
   (a) cultivating the isolated *Bacillus* transformant of paragraph [29]; wherein the heterologous polynucleotide encodes the polypeptide; and
   (b) recovering the polypeptide.

[56] A method of producing a fermentation product, comprising:
   (a) cultivating the isolated *Bacillus* transformant of paragraph [29]; wherein the heterologous polynucleotide encodes a polypeptide of the fermentation pathway and wherein the transformant is capable of producing the fermentation product; and
   (b) recovering the fermentation product.

[57] The method of paragraph [56], wherein the fermentation product is an alcohol, an alkane, a cycloalkane, an alkene, an amino acid, a gas, an isoprenoid, a ketone, an organic acid, or a polyketide.
Claims

What is claimed is:

1. An isolated mutant Bacillus strain, comprising disruption to an endogenous mecA gene and disruption to an endogenous sinl gene.

2. The isolated mutant of claim 1, wherein the mutant has improved transformation efficiency compared to the parent Bacillus strain that lacks disruption to the endogenous mecA gene and lacks disruption to the endogenous sinl gene, when cultivated under identical conditions.

3. The isolated mutant of claim 1 or 2, wherein the mutant is capable of producing at least 10-fold (e.g., at least 100-fold, at least 1000-fold, at least 10000-fold, or at least 100000-fold) more transformants compared to the parent Bacillus strain that lacks disruption to the endogenous mecA gene and lacks disruption to the endogenous sinl gene, when cultivated under identical conditions.

4. The isolated mutant of any one of claims 1-3, wherein the Bacillus strain is selected from Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus brevis, Bacillus circulans, Bacillus clausii, Bacillus coagulans, Bacillus firmus, Bacillus lautus, Bacillus lentus, Bacillus licheniformis, Bacillus megaterium, Bacillus pumilus, Bacillus stearothermophilus, Bacillus subtilis, or Bacillus thuringiensis.

5. The isolated mutant of claim 4, wherein the Bacillus strain is a Bacillus licheniformis strain.

6. The isolated mutant of claim 4, wherein the Bacillus strain is a Bacillus subtilis strain.

7. The isolated mutant of any one of claims 1-6, wherein the endogenous mecA gene (a) encodes for a polypeptide having at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 2 or 16; (b) comprises a coding sequence that hybridizes under at least low, medium, medium-high, high, or very high stringency conditions with the full-length complementary strand of SEQ ID NO: 1 or 15; or (c) comprises a coding sequence that has at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 1 or 15.
8. The mutant of any one of claims 1-7, wherein the endogenous mecA gene encodes a polypeptide comprising or consisting of SEQ ID NO: 2 or 16.

9. The mutant of any one of claims 1-8, wherein the coding sequence of the endogenous mecA gene comprises or consists of SEQ ID NO: 1 or 15.

10. The mutant of any one of claims 1-9, wherein disruption to the endogenous mecA gene occurs in the coding sequence and/or promoter sequence.

11. The mutant of any one of claims 1-10, wherein the endogenous mecA gene is inactivated.

12. The mutant of any one of claims 1-11, wherein the endogenous sinl gene (a) encodes for a polypeptide having at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 4 or 18; (b) comprises a coding sequence that hybridizes under at least low, medium, medium-high, high, or very high stringency conditions with the full-length complementary strand of SEQ ID NO: 3 or 17; or (c) comprises a coding sequence that has at least 60%, e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, at least 99%, or 100% sequence identity to SEQ ID NO: 3 or 17.

13. The mutant of any one of claims 1-12, wherein the endogenous sinl gene encodes a polypeptide comprising or consisting of SEQ ID NO: 4 or 18.

14. The mutant of any one of claims 1-13, wherein the coding sequence of the endogenous sinl gene comprises or consists of SEQ ID NO: 3 or 17.

15. The mutant of any one of claims 1-14, wherein disruption to the endogenous sinl gene occurs in the coding sequence and/or promoter sequence.

16. The mutant of any one of claims 1-15, wherein the endogenous sinl gene is inactivated.

17. A method for obtaining the isolated Bacillus mutant of any one of claims 1-16, comprising:
   (a) cultivating a parent Bacillus strain;

- 43 -
(b) disrupting an endogenous mecA gene and an endogenous sinI gene in the parent *Bacillus* strain of (a); and
(c) isolating the mutant strain resulting from (b).

18. A method for obtaining an isolated *Bacillus* transformant, comprising:
   (a) cultivating the isolated *Bacillus* mutant of any one of claims 1-16;
   (b) transforming a heterologous polynucleotide into the *Bacillus* mutant of (a); and
   (c) isolating the transformant strain resulting from (b).

19. An isolated *Bacillus* transformant produced by the method of claim 18.

20. A method of producing a polypeptide, comprising:
   (a) cultivating the isolated *Bacillus* transformant of claim 19; wherein the heterologous polynucleotide encodes the polypeptide; and
   (b) recovering the polypeptide.

21. A method of producing a fermentation product, comprising:
   (a) cultivating the isolated *Bacillus* transformant of claim 20; wherein the heterologous polynucleotide encodes a polypeptide of the fermentation pathway and wherein the transformant is capable of producing the fermentation product; and
   (b) recovering the fermentation product.
Fig. 1
Fig. 2
EIERNETVKFYISHYGDIE
ATGGAAATCGAAGAATAACACGACACCGGTTAAGTTTATATTCTACGGTGATATCGA
DRGFDREREIWYNTERSEELFW
GACCGCGGTTGACAGAGAAAGAAAATTGTGACAAATCGAGACGCGATGAAAGAGCTTTTTGG
EMMDEVHEEEEFAVEGGLWIQ
CAGAATGGATGCAAGTGCACGAGAAGAAGAAGAGTTTGTGCCGTGAGAAGGCCCCCTTTTGGATCAA
VQALDKGEIIIVTRAQLSKDG
GTGCAGGGCCCTTGAGCAAGAGTTGGAATCATTGTGACAAGAGCTCAGTTGTGCAAAAGACGGA
QKLELPIEDKKQHVAEESLD
CAAAAGCTGAACGCGATTCTCGAAGATAAAAAACAGCATGTCAGAGAAAGAAGCTTTTGAT
ALDDDFQKEEQAEEQKQLQFVL
GCTTTGCTTTGAGCAAGCCTTGCACAAAGAAAGAGACGAGCAGAAGACAAACTGCAGTTTGTGTTTTA
KFDFDFEDLISLSKMSVSQCT
AAAAATTGAGAGTTGGAATATTAAATCTCAGTGC AAAATATTGTGTCAGGCGTTGCAAAACG
TLYSHENRYYLFDNFELPDE
ACATTGTACCTCATGAAAACCCCTATTATTTATTTCCGTGGATTTCAATGACTGCCTGTGAA
EVENQLSILLEYASES KMTH
GAGGTGAAAACCCAGCTGAGCATTCTGCTGAAATATGCCTCAGAATCAAAGATGACGATTTCAT
MLKEYGKLIADHALHTIKKH
ATCCTGAAAGAGATAGTTGCAAGCGGATCATGCTTTCATACAAATAAAGAACAC
FA*
TTTGCATAA

Fig. 3

SUBSTITUTE SHEET (RULE 26)
Fig. 4
Fig. 5

SUBSTITUTE SHEET (RULE 26)
MKNAKQEHFELDQEWVELMVE
1 ATGAAGAATGCAAAAACAAGAGCACCCTTGAATTTGGATCAAGAATGGGTTGAAATTATGGTGGA
AKEANISPEEIRKYLQLNKKS
64 GCCAAGAGGCAATATCAGCCCGGAAGAATACGAAAATATTTACTTTAAAAACAAAAAGTCT
AHPGPAARSHTVNPF*
127 GCTCATCTTGGTCGGCGAGCCAGAAGTCATACCGTAAATCTTTTCGTA

Fig. 6
TaHy9 competent cells
spizL 1% xylose, 37C, 250 rpm

Fig. 7
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K14/32
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC.

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched.

Electronic database consulted during the international search (name of database and, where practical, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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[ ] Further documents are listed in the continuation of Box C.
[ ] See patent family annex.

"X" document defining the general state of the art which is not considered to be of particular relevance.
"E" earlier application or patent but published on or after the international filing date.
"L" document which may throw doubts on priority claim(s) or on the correctness of the filing date of another document, or on any other special reason (as specified).
"O" document referring to an oral disclosure, use, exhibition or other means.
"P" document published prior to the international filing date but later than the priority date claimed.

Date of actual completion of the international search: 22 November 2013

Date of mailing of the international search report: 03/12/2013

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Fax: (+31-70) 340-3016

Authorized officer

Del eu, Laurent
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