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(52) **U.S. Cl.**
CPC **C12N 15/902** (2013.01); **C12Q 1/02**
(2013.01); **C07K 7/00** (2013.01)(57) **ABSTRACT**

The present invention relates to a method for transforming a strain of the *Lactococcus* genus through natural competence. The present invention further relates to strains obtained or obtainable by said method. The present invention also relates to a method for identifying a strain of the *Lactococcus* genus which is transformable through natural competence.

Specification includes a Sequence Listing.

		Dairy / Model	Dairy	Plant	Dairy / Model	Maize
		MG1363	SK11	KW2	IL1403	SL12651 SL12653
Reg	<i>comX</i>	+	*	+	+	+
Pilus assembly	<i>comGA</i>	+	Tn	+	+	+
	<i>comGB</i>	+	*	+	+	+
	<i>comGC</i>	+	+	+	+	+
	<i>comGD</i>	+	*	+	+	+
	<i>comGE</i>	+	+	+	+	+
	<i>comGF</i>	+	+	+	+	+
	<i>comGG</i>	+	+	+	+	+
	<i>comC</i>	+	*	+	+	+
DNA uptake	<i>comFA</i>	+	*	+	+	+
	<i>comFC</i>	+	+	+	+	+
	<i>comEA</i>	+	*	+	+	+
	<i>comEC</i>	*	Tn	+	+	+
DNA protection & recombination	<i>ssbB</i>	+	+	+	+	+
	<i>coiA</i>	*	+	+	+	+
	<i>dprA</i>	+	+	+	*	+
	<i>recA</i>	+	+	+	+	+

FIG. 1

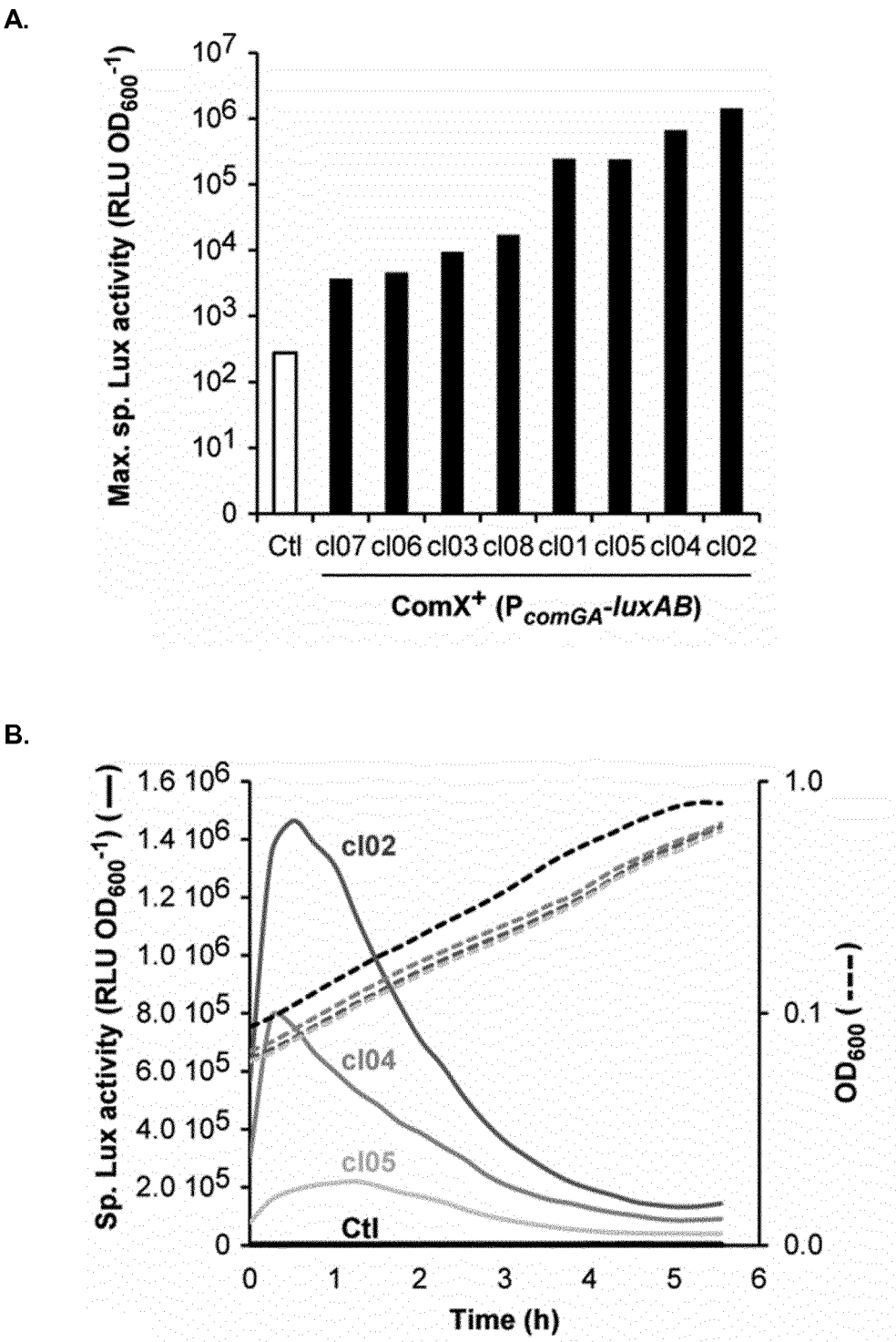
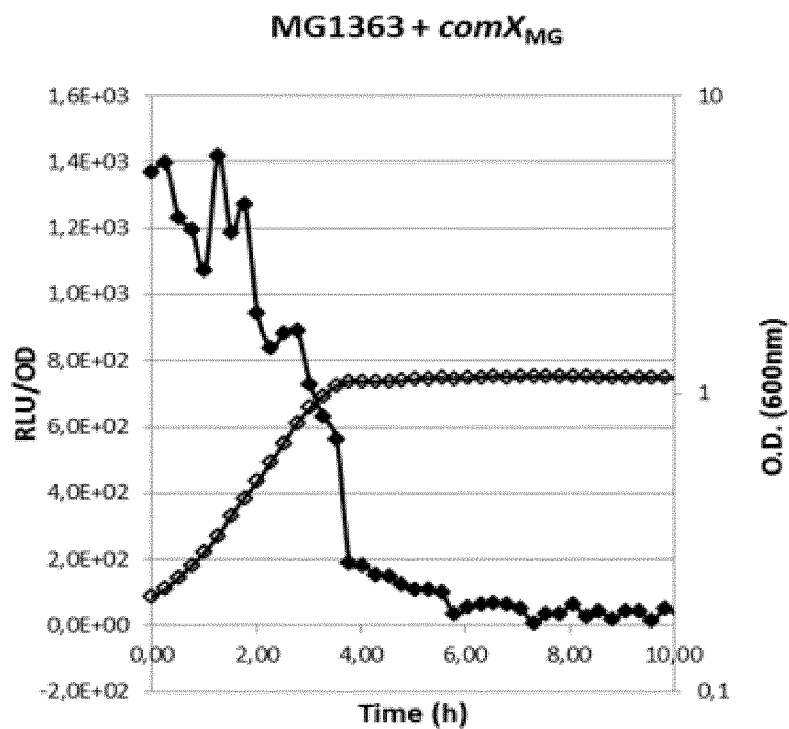


FIG. 2

C.



D.

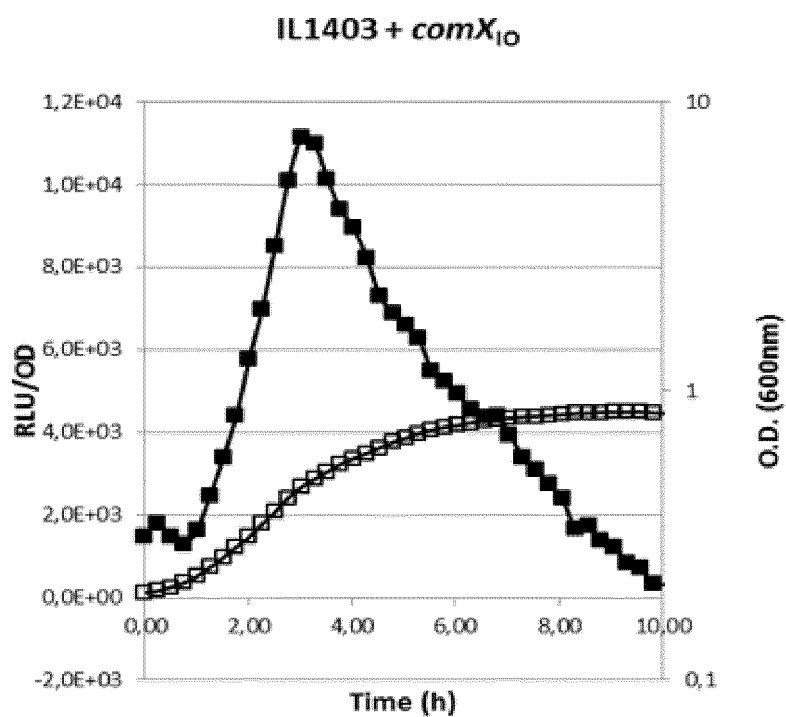


FIG. 2

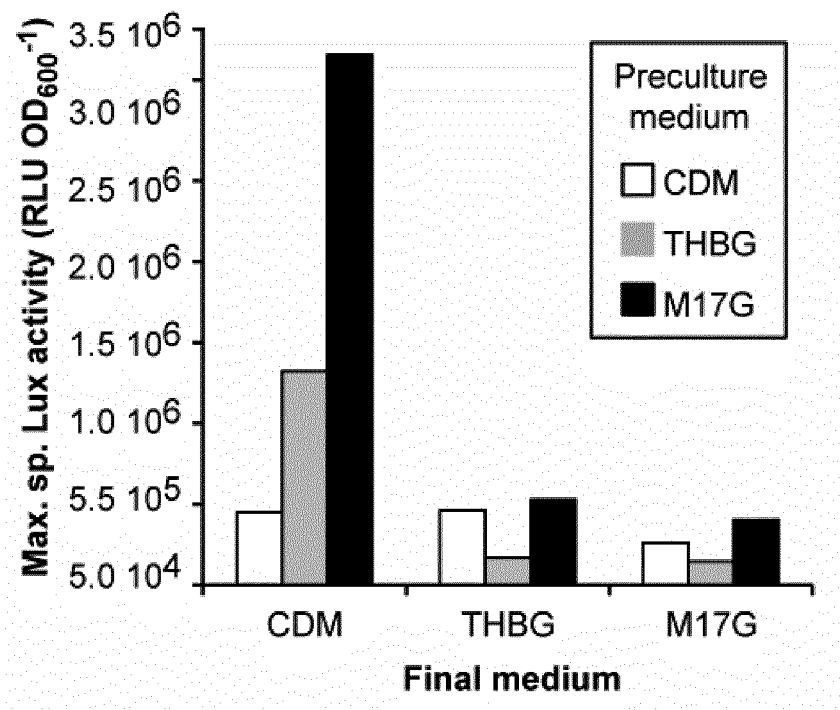


FIG. 3

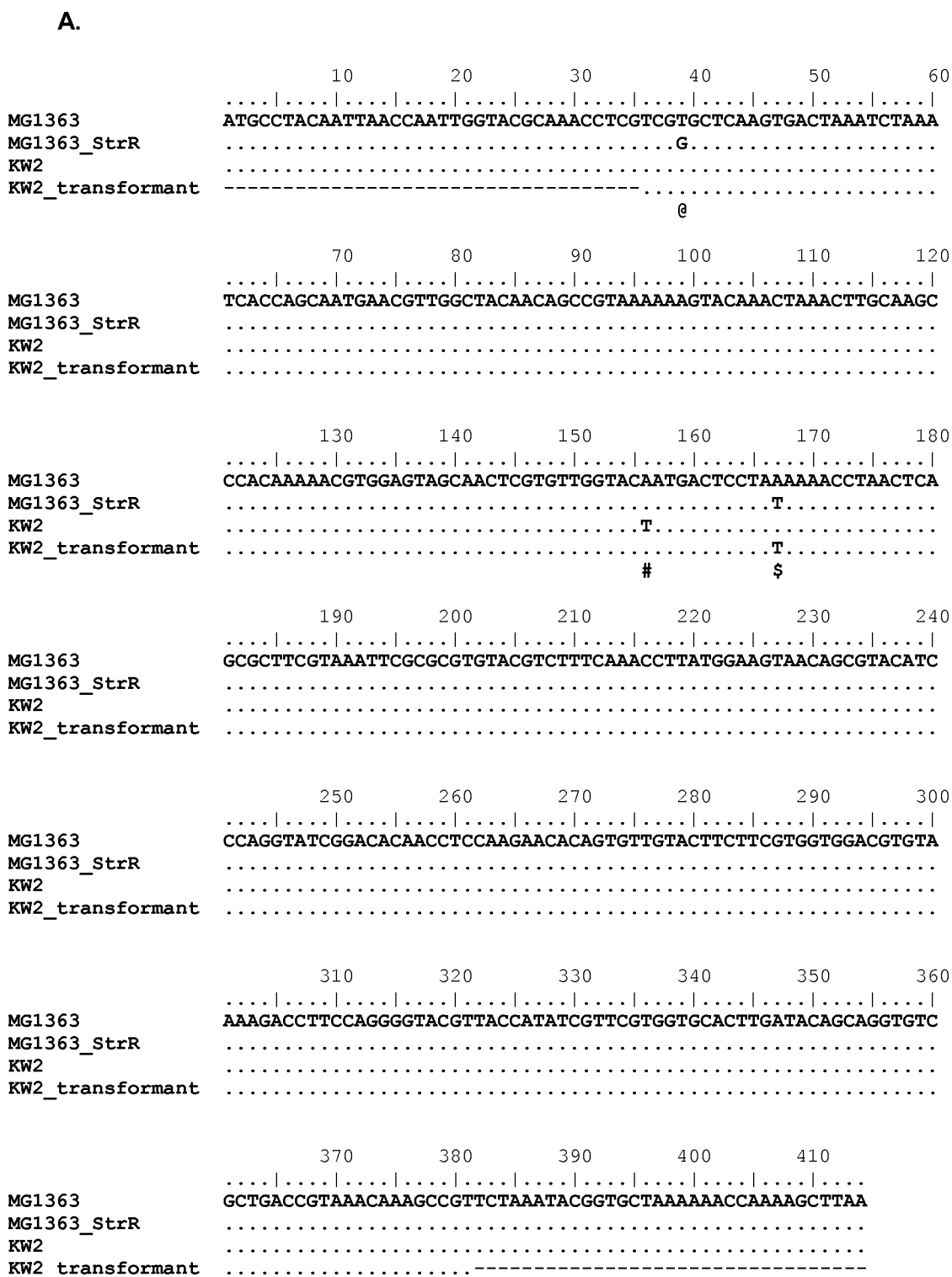


FIG. 4

B.

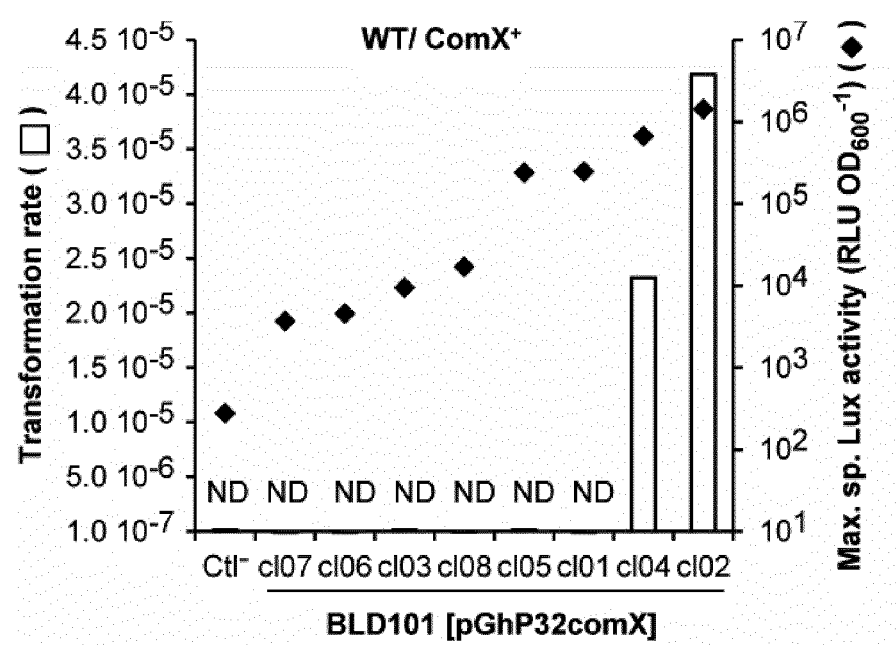


FIG. 4

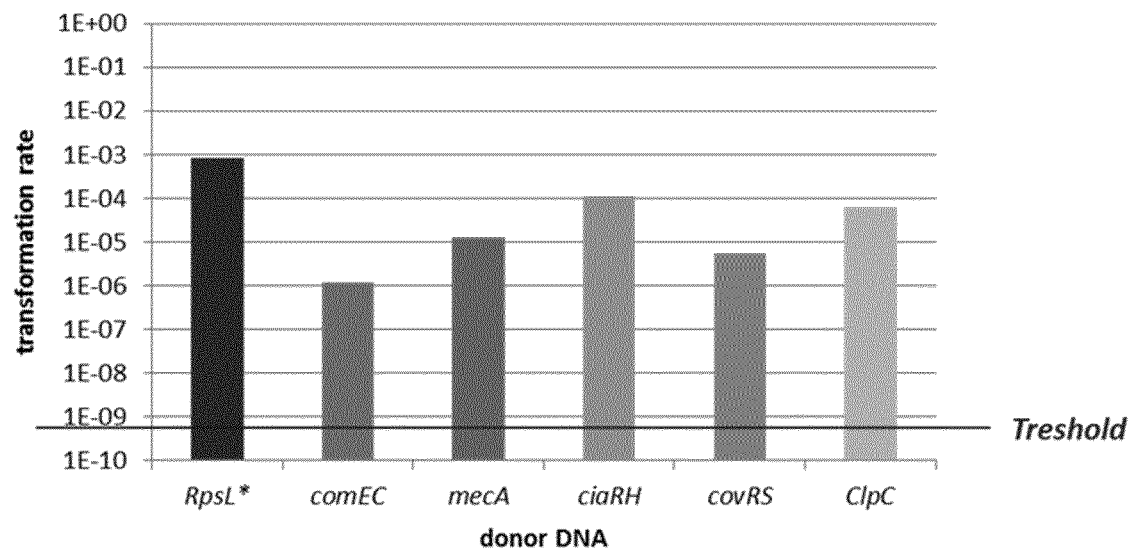


FIG. 5

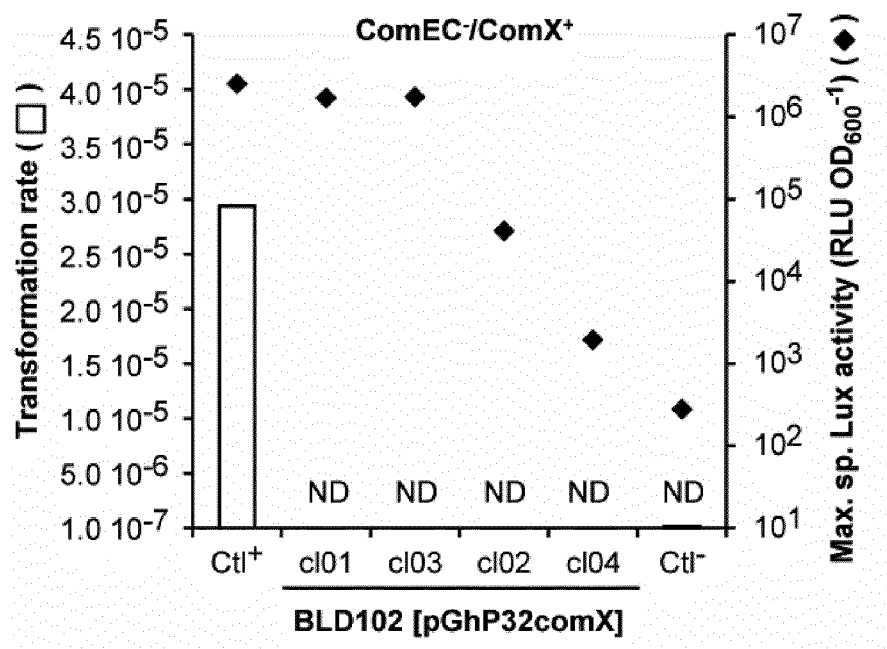


FIG. 6

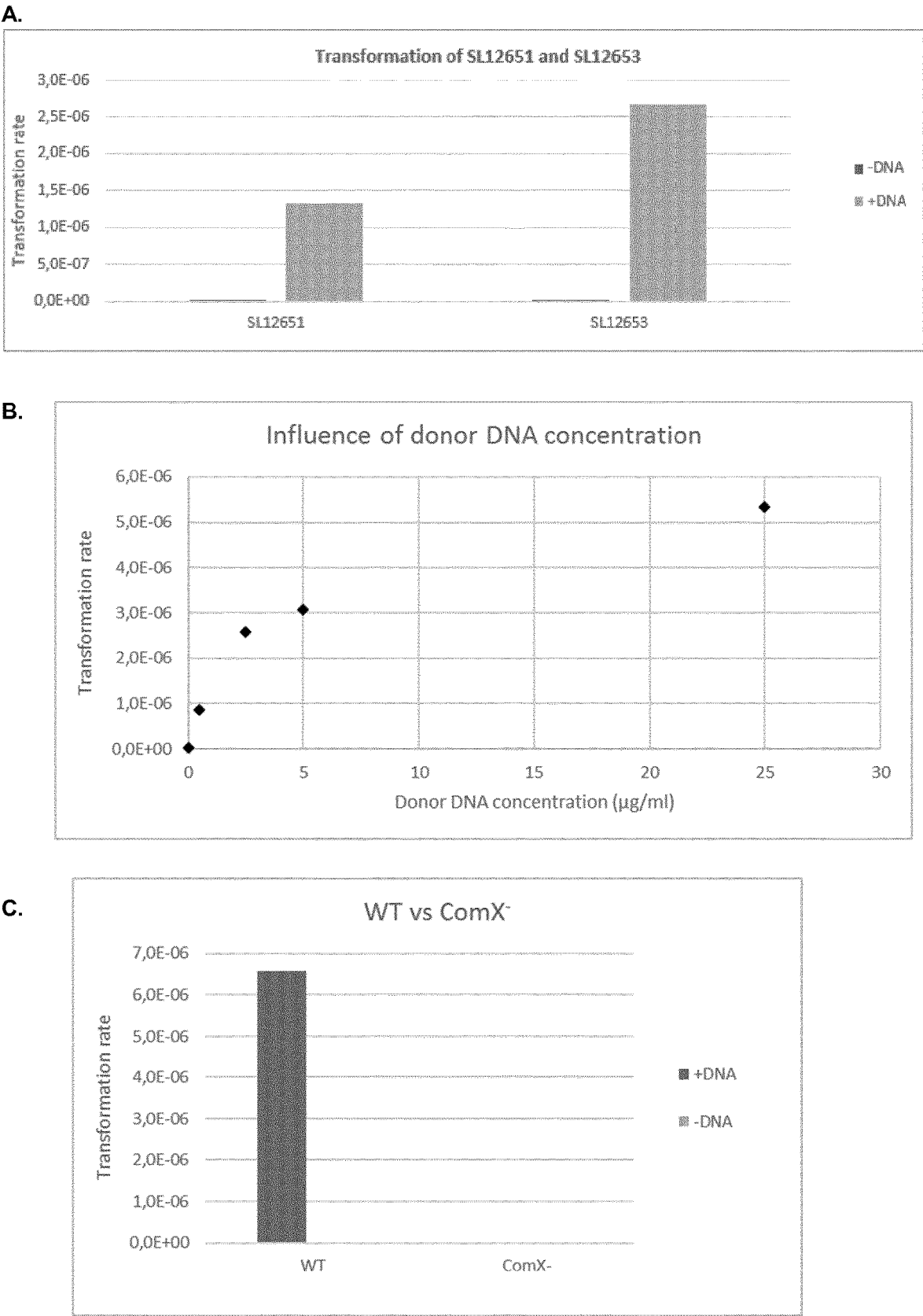


FIG. 7

METHODS AND STRAIN

FIELD OF THE INVENTION

[0001] The present invention relates to a method for transforming a strain of the *Lactococcus* genus through natural competence. The present invention further relates to strains obtained or obtainable by said method. The present invention also relates to a method for identifying a strain of the *Lactococcus* genus which is transformable through natural competence.

BACKGROUND TO THE INVENTION

[0002] *Lactococcus lactis* is one of the most important lactic acid bacteria used in the dairy industry, in particular as a main dairy starter species in various cheese preparations (e.g. gouda, cheddar, brie, parmesan, roquefort) and fermented milk products (e.g. buttermilk, sour cream). Other applications of *L. lactis* bacteria include as a host for heterologous protein production or as a delivery platform for therapeutic molecules. While the growth and fermentation properties of *L. lactis* have been gradually improved by selection and classical methods, there is great potential for further improvement through natural processes or by genetic engineering. Of particular interest are methods to naturally transform *L. lactis* without the use of genetic engineering, thereby generating new non-GMO strains with useful industrial properties.

[0003] *Lactococcus raffinolactis* is present in a wide range of environments, such as foods (meat, fish, milk, vegetable), animals, and plant materials. In the dairy environment, this species has been found in raw milks (cow, ewe, goat, and camel), natural dairy starter cultures, and a great variety of cheeses. The prevalence of this bacterium in foods even if with a “nondominant” status compared to other lactococci could make it a candidate for future development of starter cultures.

[0004] DNA acquisition by natural transformation is widespread among prokaryotes and has been identified in over 80 species. Various functions are attributed to competence for natural transformation: genome plasticity, DNA repair, and/or nutrition. In Gram-positive bacteria, competence for natural transformation has been well-characterized in *Bacillus subtilis* and in various species of the genus *Streptococcus* (e.g. *S. pneumoniae*, *S. mutans*, and *S. thermophilus*).

[0005] In streptococci, competence for DNA transformation is induced in response to secreted signalling peptides referred to as competence pheromones/alarmones. The production of this class of cell-to-cell communication molecules is initiated in response to specific environmental stresses or conditions and allows the coordination of physiological functions (e.g. competence, predation, biofilm formation). Above a threshold concentration, competence pheromones activate the master regulator ComX (alternative sigma factor σ^X), which ultimately leads to a transcriptional reprogramming of cells (globally known as late competence phase) including the induction of genes strictly required for DNA transformation. ComX binds to a specific DNA sequence named Com-box or Cin-box, which is located at least in the vicinity of promoters of late competence (com) genes/operons responsible for DNA uptake (e.g.; comG, comF and comE operons), DNA protection (e.g. ssb) and DNA recombination (e.g. recA, dprA, coiA), and positively controls their expression.

[0006] The early steps leading to competence activation (early competence phase) differs among bacteria. In streptococci, two major peptide-based signaling pathways—i.e. ComCDE and ComRS—have been identified so far. In mitis and anginosus groups of streptococci (*S. pneumoniae* as paradigm), the competence signaling peptide (CSP, or mature ComC) triggers a phosphorylation cascade mediated by the two-component system ComD-ComE, leading to the transcriptional activation of comX. In *salivarius*, *mutans*, *pyogenes*, *bovis* and suis groups of streptococci, another regulation mechanism is operational (*S. thermophilus* as paradigm). This system involves the ComX-induction peptide (XIP, or mature ComS) which is internalized by the oligopeptide transporter Opp, binds to and activates the regulator ComR, and in turn induces comX transcription.

[0007] Orthologues of comX and of all late com genes essential for natural transformation have been identified in the genome of *L. lactis*, although some are present as putative pseudogenes in different strains (Wyda et al., 2006).

[0008] Specific growth conditions have been reported to activate com genes in *Lactococcus lactis*. For example, the promoter of comX was shown to be induced during cheese-making conditions in strain MG5267 (an MG1363 derivative) which belongs to the subspecies *cremoris* (Bachmann et al. 2010).

[0009] In the *L. lactis* subspecies (subsp.) *lactis*, carbon starvation was shown to activate six late com genes in strain IL1403 of dairy origin (i.e. comX, comEA, comGA, comGB, radA, and nucA) and most of the late essential com genes in strain KF147 of plant origin (i.e. comX, comC, coiA, and operons comG, comE, comF) (Ercan et al., 2015). However, when the authors attempted to validate functional natural transformation in KF147, they were unsuccessful.

[0010] Wyda et al. reported that all the well-established late genes/operons display an upstream and conserved Com-box, suggesting that they are similarly controlled by ComX as reported in streptococci. However, the authors did not comment on whether comX over-expression in IL1403 induced natural competence. Indeed, the authors neither report any experiment evaluating natural competence in this strain nor suggest any experimental conditions appropriate for inducing natural competence. Thus, as noted in the recent literature (see Ercan et al., 2015) [i.e., 9 years after Wyda et al.], there is no experimental evidence for successful transformation of any species of the genus *Lactococcus* by natural competence, and even less of IL1403.

[0011] Accordingly, there remains a need for a method for naturally transforming *Lactococcus* strains using natural competence. In addition, since some strains of the *Lactococcus* genus may not encode a full set of functional late com genes, there is a need for a method for identifying *Lactococcus* strains which can be transformed by natural competence.

SUMMARY OF THE INVENTION

[0012] In a first aspect, the present invention provides a method for transforming a strain of the *Lactococcus* genus with an exogenous DNA polynucleotide comprising the steps of:

[0013] (a) providing a strain of the *Lactococcus* genus, wherein said strain is transformable through natural competence;

- [0014] (b) modulating the production of a ComX protein in said strain;
- [0015] (c) contacting said strain of step (b) with an exogenous DNA polynucleotide in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and
- [0016] (d) selecting a strain which has integrated the exogenous DNA polynucleotide into its genome.
- [0017] In one embodiment, the step of modulating the production of a ComX protein is performed by expressing a comX gene in said strain or increasing the expression of a comX gene in said strain.
- [0018] In a further embodiment, the comX gene is an exogenous comX gene. Said exogenous comX gene may be transferred into said strain by conjugation, transduction, or transformation. Said exogenous comX gene may be operably linked to transcription regulator(s).
- [0019] In an alternative embodiment, said comX gene is the endogenous comX gene of said strain.
- [0020] In one embodiment, when said comX gene is the endogenous comX gene of said strain, the method comprises:
- [0021] (a) providing a strain of the *Lactococcus* genus, wherein said strain is transformable through natural competence;
- [0022] (b) modulating the production of a ComX protein, by expressing the endogenous comX gene or increasing the expression of the endogenous comX of said strain;
- [0023] (c) contacting said strain of step (b) with an exogenous DNA polynucleotide in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and
- [0024] (d) selecting a strain which has integrated the exogenous DNA polynucleotide into its genome,
- [0025] wherein step (c) is carried out after step (b) or wherein step (b) and step (c) are carried out simultaneously.
- [0026] In some embodiments, said ComX protein has the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, or has at least 90% identity or at least 90% similarity to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20 or SEQ ID NO:22. In some embodiments, said ComX protein has the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, or has at least 90% identity or at least 90% similarity to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6.
- [0027] In some embodiments, said comX gene has the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, or has at least 90% identity to the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19 or SEQ ID NO:21.
- [0028] In some embodiments, said comX gene has the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, or has at least 90% identity to the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5.
- [0029] In some embodiments, the medium of step (c) is a chemically defined medium. In a preferred embodiment the chemically defined medium (CDM) comprises 0.5 g/L NH₄Cl, 9.0 g/L KH₂PO₄, 7.5 g/L K₂HPO₄, 0.2 g/L MgCl₂, 5 mg/L FeCl₂, 50 mg/L CaCl₂, 5 mg/L ZnSO₄, 2.5 mg/L CoCl₂, 0.05 g/L tyrosine, 0.1 g/L asparagine, 0.1 g/L cysteine, 0.1 g/L glutamine, 0.1 g/L isoleucine, 0.1 g/L leucine, 0.1 g/L methionine, 0.1 g/L tryptophan, 0.1 g/L valine, 0.1 g/L histidine, 0.2 g/L arginine, 0.2 g/L glycine, 0.2 g/L lysine, 0.2 g/L phenylalanine, 0.2 g/L threonine, 0.3 g/L alanine, 0.3 g/L proline, 0.3 g/L serine, 10 mg/L paraaminobenzoic acid, 10 mg/L biotin, 1 mg/L folic acid, 1 mg/L nicotinic acid, 1 mg/L pantothenic acid, 1 mg/L riboflavin, 1 mg/L thiamine, 2 mg/L pyridoxine, 1 mg/L cyanocobalamin, 5 mg/L orotic acid, 5 mg/L 2-deoxythymidine, 5 mg/L inosine, 2.5 mg/L dl-6,8-thioctic acid, 5 mg/L pyridoxamine, 10 mg/L adenine, 10 mg/L guanine, 10 mg/L uracil, 10 mg/L xanthine, and 5 g/L glucose.
- [0030] In some embodiments, prior to step (c) said strain is incubated in a pre-culture medium, preferably wherein the pre-culture medium is a complex medium, more preferably wherein the pre-culture medium is M17G or THBG.
- [0031] In some embodiments of the present invention, said strain is incubated with the exogenous DNA polynucleotide for around 4 to 8 hours at around 30° C. and said medium of step (c) is supplemented with an osmo-stabilizer, preferably wherein the osmo-stabilizer is glycerol or mannitol, more preferably wherein the osmo-stabilizer is 5% [v/v] glycerol or 5% [w/v] mannitol.
- [0032] In some embodiments, said exogenous DNA polynucleotide is from a strain of the *Lactococcus lactis* species.
- [0033] In some embodiments, said exogenous DNA polynucleotide is from a strain of the *Lactococcus raffinolactis* species.
- [0034] In some embodiments, said strain of step (a) is a *Lactococcus lactis* subsp. *cremoris* strain.
- [0035] In another aspect, the present invention provides a strain of the *Lactococcus* genus obtained or obtainable by the method of the first aspect of the present invention.
- [0036] In one embodiment, said strain of the *Lactococcus* genus is a strain of the *Lactococcus lactis* or *Lactococcus raffinolactis* species.
- [0037] In a further aspect, the present invention provides a method for identifying a strain of the *Lactococcus* genus which is transformable through natural competence comprising the steps of:
- [0038] (a) providing a strain of the *Lactococcus* genus species;
- [0039] (b) transforming said strain with a plasmid expressing a comX gene having at least 90% identity, preferably having 100% identity, to the endogenous comX gene of said strain;
- [0040] (c) contacting said strain obtained in step (b) with an exogenous DNA polynucleotide encoding a marker gene in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and
- [0041] (d) determining the rate of recombination events;

[0042] wherein a rate of at least 1×10^{-6} transformants per μg of DNA is indicative of a strain which is transformable through natural competence.

[0043] In a particular embodiment of method for transforming a strain of the *Lactococcus* genus of the present invention, said strain of step (a) is identified using the method for identifying a strain of the *Lactococcus* genus which is transformable through natural competence according to the present invention. In some embodiments of the present invention, said strain of step (a) is identified using Assay A.

DESCRIPTION OF THE DRAWINGS

[0044] FIG. 1: Table showing the status of genes involved in natural competence for *L. lactis* strains MG1363, SK11, KW2, IL1403, SL12651 and SL12653.

[0045] Late com genes in the complete genomes of strains MG1363, SK11, and KW2 of *Lactococcus lactis* subsp. *cremoris* and of strain IL1403, SL12651 and SL12653 of *Lactococcus lactis* subsp. *lactis*. Origin is indicated above strain names. Gene-associated function in DNA transformation is indicated on the left. Reg. denotes regulation. The complete and incomplete status of late genes is based on blastp and tblastn homology searches (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) using orthologues of *S. pneumoniae* TIGR4 and *S. thermophilus* LMD-9 and default parameters. +denotes the presence of a complete gene; * denotes the presence of an incomplete gene due to nucleotide(s) exchange, insertion or deletion resulting in a premature stop codon; and Tn denotes a disrupted gene by the insertion of at least one transposon.

[0046] FIG. 2: Graphs displaying the results of luciferase assays which demonstrate the activation of a reporter construct comprising the late promoter P_{comGA} driven by constitutive comX overexpression

[0047] (A) Maximum specific luciferase (Lux) activity (RLU OD_{600}^{-1}) emitted by eight independent clones (cl01 to cl08) of the KW2-derived reporter strain (BLD101, $P_{comGA[MG]}-luxAB$) carrying plasmid pGhP32comX_{MG} compared to the control strain (Ctl) carrying the empty vector pG⁺host9. (B) Kinetics of specific Lux activity (solid line) during growth (RLU/OD_{600} ; dotted line) for the control strain (Ctl; black lines) and three selected clones (BLD101 [pGhP32comX_{MG}], cl02, cl04 and cl05; gray lines). (C) Kinetics of specific luciferase activity (closed symbols) during growth (RLU/OD_{600} ; open symbols) of the MG1363+pGhP32comX_{MG}- $P_{comGA[MG]}-lux$, grown in M17G at 30° C. (D) Kinetics of specific luciferase activity (closed symbols) during growth (RLU/OD_{600} ; open symbols) of IL1403+pGhP32comX_{IO}- $P_{comGA[IO]}-lux$ strains, grown in M17G at 30° C.

[0048] FIG. 3: Graphs displaying the results of luciferase assays which demonstrate the impact of growth medium on P_{comGA} activation

[0049] Maximum specific Lux activity of BLD101 [pGhP32comX_{MG}] cl02 grown in different final culture media (CDM, THBG, and M17G) according to preculture conditions (CDM, THBG, and M17G). Overnight precultures were 10-fold diluted in the pre-culture medium and grown for 2 hours. Then, cells were washed twice in distilled water and the OD_{600} was adjusted to 0.05 in the final growth medium before measuring growth and luciferase activity. One representative experiment of two independent replicates.

[0050] FIG. 4: Results of a transformation assay implemented on a *L. lactis* subsp. *cremoris* KW2 constitutively expressing comX contacted with a DNA consisting of a mutated allele of the rpsL gene as exogenous DNA polynucleotide

[0051] (A) Alignment of the rpsL gene sequences of strain MG1363, a spontaneous streptomycin-resistant clone of strain MG1363, strain KW2 and a KW2-derived transformant obtained using the method of the invention (partial sequence). The arabase, pound and dollar signs below the alignment indicate the positions of nucleotide differences existing between the rpsL sequences. The dollar sign at position 167 indicates the point mutation (A→T; strA1 allele) responsible for the streptomycin-resistance phenotype; the pound sign at position 156 highlights a nucleotide that is naturally different between MG1363 and KW2 (T in KW2, A in MG1363); the arabase sign at position 39 indicates a silent nucleotide substitution (T→G) which is found in the streptomycin-resistant clone derived from MG1363. (B) DNA transformation with the strA1 allele was assessed for *L. lactis* strains constitutively expressing ComX. Transformation rate (white bars) and maximum specific luciferase (Lux) activity (black diamonds, RLU OD_{600}^{-1} , as reported in FIG. 2) of eight clones (cl01 to cl08) of the reporter strain (BLD101, $P_{comGA[MG]}-luxAB$) carrying plasmid pGhP32comX_{MG} compared to the negative control strain (Ctl-) carrying the empty vector (BLD101 [pG⁺host9]).

[0052] FIG. 5: Graphs displaying the results of transformation rate of the KW2 derivative BLD101 [pGhP32comX_{MG}] obtained with overlap PCR products (comEC, mecA, ciaRH, covRS and clpC) and strA1 (rpsL*)-donor DNA.

[0053] The threshold represents the theoretical transformation rate to obtain only one transformant.

[0054] FIG. 6: Graphs depicting the results of transformation assays for a *L. lactis* subsp. *cremoris* deleted in its comEC gene and constitutively expressing comX.

[0055] DNA transformation with the strA1 allele was assessed for *L. lactis* strains constitutively expressing comX. Transformation rate (white bars) and maximum specific luciferase (Lux) activity (RLU OD_{600}^{-1}) of four clones (cl01 to cl04) of the ComEC-deficient reporter strain (BLD102, $P_{comGA[MG]}-luxAB$) carrying plasmid pGhP32comX_{MG} compared to the positive (Ctl⁺, BLD101 [pGhP32comX_{MG}] cl02) and negative (Ctl⁻, BLD101 [pG⁺host9]) control strains. Transformability was assessed according to the standard protocol described in Materials and Methods using strA1-carrying PCR products as donor DNA. ND denotes a transformation rate below the detection level of spontaneous Str^r mutants ($<10^{-7}$). One representative experiment of two independent replicates.

[0056] FIG. 7: Graphs displaying natural competence in *Lactococcus lactis* subsp. *lactis* SL12651 and 12653 strains.

[0057] (A) Transformation rate of *L. lactis* subsp. *lactis* SL12651 and 12653 strains in M17G medium, with rpsL* donor DNA (+DNA) or without donor DNA (-DNA); (B) DNA transformation with increasing initial concentration of donor DNA assessed in SL12653 strain; (C) Comparison of transformation rates between wild-type (WT) SL12653 strain and a SL12653 strain deleted for the comX gene (ComX-); transformation rate of three clones of the ComX-deficient strain compared to the WT strain, in presence (+DNA) or in absence (-DNA) of donor DNA.

DETAILED DESCRIPTION

[0058] The present invention is based on the observation that overexpression of ComX in a strain of the *Lactococcus* genus allowed to transform this strain by natural competence. Using this approach a *L. lactis* strain was generated by natural transformation with an exogenous DNA polynucleotide. Importantly, these results are the first demonstration of transformation of a *L. lactis* strain by natural competence. Further, existence of natural competence in the *Lactococcus* genus has been confirmed in two strains of the *Lactococcus raffinolactis* species and two *Lactococcus lactis* species.

[0059] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, biochemistry, microbiology, bacteriology, and related fields, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature.

[0060] Thus, the present invention provides a method for transforming a strain of the *Lactococcus* genus with an exogenous DNA polynucleotide comprising the steps of:

[0061] (a) providing a strain of the *Lactococcus* genus, wherein said strain is transformable through natural competence;

[0062] (b) modulating the production of a ComX protein in said strain;

[0063] (c) contacting said strain of step (b) with an exogenous DNA polynucleotide in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and

[0064] (d) selecting a strain which has integrated the exogenous DNA polynucleotide into its genome.

[0065] As detailed below, step (b) and step (c) can be carried out sequentially [i.e., step (b) and then step (c)] or in another embodiment step (b) and step (c) can be carried out simultaneously.

Lactococcus Genus

[0066] The present invention relates to a method for transforming a strain of the *Lactococcus* genus, a Gram-positive bacterium. *Lactococcus* strains are known as lactic acid bacteria (LAB) for their ability to convert carbohydrate to lactic acid. A strain of the *Lactococcus* genus and *Lactococcus* strain are used herein interchangeably.

[0067] The *Lactococcus* genus comprises, but is not limited to the following species: *Lactococcus chungangensis*, *Lactococcus fujiensis*, *Lactococcus garvieae*, *Lactococcus lactis*, *Lactococcus piscium*, *Lactococcus plantarum* and *Lactococcus raffinolactis*. Any strain of one of these species may be used in the current invention, provided that this strain is transformable through natural competence as defined herein.

[0068] In a particular embodiment, said strain of the *Lactococcus* genus of step (a) is a strain of the *Lactococcus lactis* species or a strain of the *Lactococcus raffinolactis* species.

Lactococcus lactis

[0069] In a particular embodiment, said strain of the *Lactococcus* genus of step (a) is a strain of the *Lactococcus lactis* species. The species *Lactococcus lactis* comprises several subspecies. Thus, when the strain of the *Lactococcus* genus of step (a) is a strain of the *Lactococcus lactis* species, said strain is selected in the group consisting of *Lactococcus*

lactis subsp. *cremoris*, *Lactococcus lactis* subsp. *hordniae*, *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *tractae*. As used herein a strain of the *Lactococcus lactis* species is understood to be a genetic variant or subtype of any *L. lactis* species or subspecies. The different *Lactococcus lactis* subspecies disclosed here, and in particular the *lactis* and the *cremoris* subspecies, are defined herein based on DNA sequences coding for 16S ribosomal RNA [Ward et al., 1998].

[0070] In a particular aspect, the present invention provides a method for transforming a strain of the *Lactococcus lactis* species with an exogenous DNA polynucleotide comprising the steps of:

[0071] (a) providing a strain of the *Lactococcus lactis* species, wherein said strain is transformable through natural competence;

[0072] (b) modulating the production of a ComX protein in said strain;

[0073] (c) contacting said strain of step (b) with an exogenous DNA polynucleotide in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and

[0074] (d) selecting a strain which has integrated the exogenous DNA polynucleotide into its genome.

[0075] In a preferred embodiment, the strain of step (a) is a *Lactococcus lactis* subsp. *cremoris* strain or a *Lactococcus lactis* subsp. *lactis* strain. Both subspecies have been identified and characterised with full genome sequences see, e.g., Wegmann et al. (2007) J. Bacteriol. 189:3256-3270 and Bolotin et al. (2001) Genome Res. 11:731-753. With regards to the dairy industry, *L. lactis* subsp. *lactis* (previously known as *Streptococcus lactis*) is preferred for making soft cheese while *L. lactis* subsp. *cremoris* (previously known as *Streptococcus cremoris*) is preferred for hard cheese production.

[0076] In a preferred embodiment, the strain of step (a) is *Lactococcus lactis* subsp. *cremoris* strain.

[0077] In another preferred embodiment, the strain of step (a) is *Lactococcus lactis* subsp. *lactis* strain.

Lactococcus raffinolactis

[0078] In a particular embodiment, said strain of the *Lactococcus* genus of step (a) is a strain of the *Lactococcus raffinolactis* species.

[0079] In a particular aspect, the present invention provides a method for transforming a strain of the *Lactococcus raffinolactis* species with an exogenous DNA polynucleotide comprising the steps of:

[0080] (a) providing a strain of the *Lactococcus raffinolactis* species, wherein said strain is transformable through natural competence;

[0081] (b) modulating the production of a ComX protein in said strain;

[0082] (c) contacting said strain of step (b) with an exogenous DNA polynucleotide in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and

[0083] (d) selecting a strain which has integrated the exogenous DNA polynucleotide into its genome.

Lactococcus plantarum

[0084] In a particular embodiment, said strain of the *Lactococcus* genus of step (a) is a strain of the *Lactococcus plantarum* species.

[0085] In a particular aspect, the present invention provides a method for transforming a strain of the *Lactococcus plantarum* species with an exogenous DNA polynucleotide comprising the steps of:

[0086] (a) providing a strain of the *Lactococcus plantarum* species, wherein said strain is transformable through natural competence;

[0087] (b) modulating the production of a ComX protein in said strain;

[0088] (c) contacting said strain of step (b) with an exogenous DNA polynucleotide in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and

[0089] (d) selecting a strain which has integrated the exogenous DNA polynucleotide into its genome.

Lactococcus piscium

[0090] In a particular embodiment, said strain of the *Lactococcus* genus of step a) is a strain of the *Lactococcus piscium* species.

[0091] In a particular aspect, the present invention provides a method for transforming a strain of the *Lactococcus piscium* species with an exogenous DNA polynucleotide comprising the steps of:

[0092] (a) providing a strain of the *Lactococcus piscium* species, wherein said strain is transformable through natural competence;

[0093] (b) modulating the production of a ComX protein in said strain;

[0094] (c) contacting said strain of step (b) with an exogenous DNA polynucleotide in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and

[0095] (d) selecting a strain which has integrated the exogenous DNA polynucleotide into its genome.

Lactococcus garvieae

[0096] In a particular embodiment, said strain of the *Lactococcus* genus of step a) is a strain of the *Lactococcus garvieae* species.

[0097] In a particular aspect, the present invention provides a method for transforming a strain of the *Lactococcus garvieae* species with an exogenous DNA polynucleotide comprising the steps of:

[0098] (a) providing a strain of the *Lactococcus garvieae* species, wherein said strain is transformable through natural competence;

[0099] (b) modulating the production of a ComX protein in said strain;

[0100] (c) contacting said strain of step (b) with an exogenous DNA polynucleotide in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and

[0101] (d) selecting a strain which has integrated the exogenous DNA polynucleotide into its genome.

Lactococcus fujiensis

[0102] In a particular embodiment, said strain of the *Lactococcus* genus of step a) is a strain of the *Lactococcus fujiensis* species.

[0103] In a particular aspect, the present invention provides a method for transforming a strain of the *Lactococcus fujiensis* species with an exogenous DNA polynucleotide comprising the steps of:

[0104] (a) providing a strain of the *Lactococcus fujiensis* species, wherein said strain is transformable through natural competence;

[0105] (b) modulating the production of a ComX protein in said strain;

[0106] (c) contacting said strain of step (b) with an exogenous DNA polynucleotide in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and

[0107] (d) selecting a strain which has integrated the exogenous DNA polynucleotide into its genome.

Lactococcus chungangensis

[0108] In a particular embodiment, said strain of the *Lactococcus* genus of step a) is a strain of the *Lactococcus chungangensis* species.

[0109] In a particular aspect, the present invention provides a method for transforming a strain of the *Lactococcus chungangensis* species with an exogenous DNA polynucleotide comprising the steps of:

[0110] (a) providing a strain of the *Lactococcus chungangensis* species, wherein said strain is transformable through natural competence;

[0111] (b) modulating the production of a ComX protein in said strain;

[0112] (c) contacting said strain of step (b) with an exogenous DNA polynucleotide in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and

[0113] (d) selecting a strain which has integrated the exogenous DNA polynucleotide into its genome.

DNA Acquisition

[0114] Bacteria may naturally acquire exogenous DNA via one of three possible mechanisms: transformation, conjugation, or transduction.

[0115] As used herein the term “transformation” refers to the uptake of exogenous genetic material (e.g. a DNA polynucleotide) from the external medium. Since transformation requires that genetic material cross the bacterial cell wall and membrane and the uptake of exogenous genetic material is energetically costly, the process is tightly regulated. Accordingly, bacterial cells may only be transformed under certain conditions. Bacterial cells which are in a transformable state are said to be competent.

[0116] Competence may be artificially induced in the laboratory, e.g. by electroporation or exposure to divalent cations (e.g. CaCl_2) and heat shock. Alternatively, some species of bacteria express a proteinaceous machinery that provides natural competence; this system of natural competence has been widely studied in streptococci.

[0117] As used herein the term “conjugation” refers to the transfer of genetic material between bacterial cells.

[0118] As used herein the term “transduction” refers to the transfer of genetic material from a virus (e.g. a bacteriophage) or a viral vector into bacterial cell.

ComX Protein

[0119] The method of the present invention comprises the step of modulating the production of a ComX protein in said strain.

[0120] ComX protein is an alternative sigma factor, also known as σ^x , which acts as master regulator for the late com genes and is responsible for transcriptional reprogramming of cells including the induction of genes strictly required for DNA transformation (Lee et al., 1989; Petersen et al. 2004).

[0121] ComX may bind to a specific target sequence (or box) termed the Com-box (or Cin-box). Com-boxes are located in the vicinity of the promoters of late competence (com) genes/operons responsible for DNA uptake (e.g., comG, comF, and comE operons), DNA protection (e.g. ssb) and DNA recombination (e.g. recA, dprA, coiA), and positively controls their expression (Campbell et al., 1998; Luo and Morrison, 2003).

[0122] The production of the ComX protein in a strain of interest may be increased relatively to an appropriate control strain, i.e., the *Lactococcus* strain in which the production of the ComX protein has not been modulated. ComX protein may be produced (expressed) following modulation as compared to an appropriate control strain, i.e., the *Lactococcus* strain in which the ComX protein is not produced.

[0123] In some embodiments, the production of the ComX protein is constitutive or inducible.

[0124] The production of ComX protein may be monitored using any method known in the art. For example, by western blotting using an antibody specific for the ComX protein. Alternatively, comX gene mRNA transcript levels may be measured by qPCR.

[0125] Alternatively, the ComX protein may be monitored using a reporter construct polynucleotide, e.g. as described in the Example 1 and Materials and Methods. The reporter construct polynucleotide may comprise genes encoding one or more reporter proteins, preferably the genes encoding the reporter proteins are operably linked to a promoter comprising a Com-box sequence. The reporter proteins may be LuxAB or Luc. Accordingly, ComX expression (and activity) may be detected and measured using a luciferase assay (Fontaine et al., 2010).

[0126] In some embodiments of the method of the present invention, the step of modulating the production of a ComX protein is performed by expressing a comX gene in said strain or increasing the expression of a comX gene in said strain. In a particular embodiment, the step of modulating the production of a ComX protein is performed by expressing a comX gene in said strain in some growth conditions, whereas said strain does not express the ComX protein outside of these growth conditions. In a particular embodiment, the step of modulating the production of a ComX protein is performed by increasing the expression of a comX gene in said strain in some growth conditions.

[0127] The comX gene may be an exogenous comX gene. As used herein an "exogenous comX gene" is understood to be a comX gene which is brought into the cytoplasm of the *Lactococcus* strain of step a), in order to be expressed. The exogenous comX gene may have the same sequence as the comX gene found in the genome of the *Lactococcus* strain of step a) or may have a different sequence from the comX gene found in the genome of the *Lactococcus* strain of step a). When different, the comX gene may be derived from a

strain of a different species, a different subspecies or a different strain of *Lactococcus*.

[0128] The exogenous comX gene may be integrated within the genome of said *Lactococcus* strain.

[0129] Alternatively, the exogenous comX gene may be located within a vector. The vector may be selected from a plasmid, a viral vector (e.g. a phage), a cosmid, or a bacterial artificial chromosome.

[0130] Said plasmid may be transferred into said *Lactococcus* strain by conjugation, transformation or transduction. Said plasmid may be auto-replicative in the transformed *Lactococcus* strain or not.

[0131] The exogenous comX gene may be operably linked to transcription regulator(s). The exogenous comX gene may be located in a linear or circular polynucleotide.

[0132] Alternatively, in some embodiments of the method of the present invention, the comX gene is the endogenous comX gene of said *Lactococcus* strain. As used herein "the endogenous comX gene of said strain" is understood to be a comX gene that is naturally present in the genome of said strain.

[0133] In some embodiments, said comX gene is a *Lactococcus* comX gene. In an embodiment, said comX gene is a *Lactococcus lactis* comX gene. In a particular embodiment, said comX gene is a *Lactococcus lactis* subsp. *lactis* comX gene. In a particular embodiment, said comX gene is a *Lactococcus lactis* subsp. *cremoris* comX gene.

[0134] The comX gene may comprise or consist of a nucleotide sequence selected from the group consisting of:

[0135] SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19; SEQ ID NO:21;

[0136] a nucleotide sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19; SEQ ID NO:21;

[0137] a variant of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21 encoding respectively a ComX protein of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20 or SEQ ID NO:22; and

[0138] a variant of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21 encoding respectively a functional ComX protein having at least 90% identity or at least 90% similarity to a ComX protein of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20 or SEQ ID NO:22.

[SEQ ID NO: 1]

ATAACATATTACTTGAAGAAGAGGATTTTAAAACTTTTTCAGAAATGAAACCTATAGTTATGAA

ATTAATGAACAACAAATTCGCATTAGAACATGAAAAATAGAGGATTATCTTCAAGAGGGGATGATTATT

- continued

TACATCTTCTATTAGAAGAGCAGAACGATGGTCAAAAGCTGCATACAAAATTTAAGGTAAAGTATCAT
CAAAGATTAATAGATGAATTAAGACGAAGTTATGCAAAGAAACGAAGCCATGACCATTTTATAGGTTT
AGATGTTTATGAATGCTCAGACTGGATAAATTCAGGTGATACTAGTCCAGATAATGAAGTGGTCTTCA
ATCATTGCTGGCAGAAGTATATGAAGGTTTGAGCGCACATTATCAAGACTTACTACTCGACAAATG
CGAGGAGAAGAACTAACTCGCATGCAACGGTATCGCCTTCGTGAAAAAATAAAGGCCATCTTATTTTC
AGAAGACGAAGAGTGA

[SEQ ID NO: 2]

MTYYLEEDFENLFSEMKP IVMKLMKQIRIRTWKIEDYLQEGMIILHLLLEEQNDGQKLHTKPKVKYH
QRLIDELRRSYAKKRSHDFIGLDVYECSDWINSGDTSPDNEVVFNHLLAEVYEGLSAHYQDLLLRQM
RGEELTRMQRYRLREKIKAILFSEDEE

[SEQ ID NO: 3]

ATGACATATTACCTGGAAGAAAATGAATTCGAAGGTTTATTTCTGGAATGAAACCAATCATCAGAAA
ATTGATGAAACAAATTCGAATCAAAGCATGGGACATAGAGGATTATTATCAAGAAGGAATGATTATTT
TGCATCACCTTTTAGAAGAAAATCACCCATCCACTAATATTATACAAAAGTTCAAAGTAAATATCAT
CAACATTTGATTGATGAACACGCCATAGCTACGCCAAAAAACGGCTTCATGACCATTTTGTAGGTCT
GGACATTTTATGAATGTTTCGGACTGGATAGATGCAGGAGGAAGTACCCCTGAAAGCGAGCTTGTGTTCA
ATCATCTTTTAGCAGAAGTTTATGAAGGATTGAGCGCCACTATCAGGAATTACTCGTGCCTCAAATG
AGAGGAGAAGAACTCACGCGAATGGAACGCTATCGGCTAAGAGAAAAAATCAAAAATATACTATTTTC
TCGAGATGATGATTAA

[SEQ ID NO: 4]

MTYYLEENEFEGFLFSGMKPIIRKLMKQIRIKAWDIEDYYQEGMIILHLLLEENHPSTNIYTKPKVKYH
QHLIDELRHSYAKKRLHDHFVGLDIYECSDWIDAGGSTPESELVFNHLLAEVYEGLSAHYQELLVRQM
RGEELTRMERYRLREKIKNILFSRDDD

[SEQ ID NO: 5]

ATGGATGACATTCAAGAAAAATACGGTTTAGAATTC AACGAATTATTCTCTGAGATGCGGCCGATAAT
TTATAAATTGATGAAGCAATTGCACATCAACACATGGGATTACGATGATTACTTCCAAGAGGGAATGA
TTACACTACATGAATTGCTGCAGAAAAATACAAATTTAGATCATGTACATACGAAATTTAAAGTGGCT
TACCATCAGCACTTAATTGACGAAATTCGCCATATTAAAGCACGAAAAAGAGGTTTGTATCAGCTCCA
TCCGATCAATGTTTATGACTGCGCAGATTGGATTGGCTCAAACCTTGCTACACCTGAAAGCGAGATAG
TTTTCAACCATCTACTAGAAGAAGTTTATGATAAACTTTCAACACACTATAAAGAACTGTTGGTAAAG
CAAAATGCATGGGGAACATCTTACGAGAATGCAGAAGTATCGTTTAAAGGAAAAAATTAAGCGATTTT
ATTTGATGAAGACTAA

[SEQ ID NO: 6]

MDDIQEKYGLFENLFSEMRPITYKLMKQLHINTWDYDDYFQEGMITLHELLQKITNLDHVHTKPKVA
YHQHLIDEIRHIKARKRGFDQLHPINVYDCADWIGSNLATPESEIVFNHLLLEEVDKLSHYKELLVK
QMHGEHLTRMQRYRLREKIKAILFDED

[SEQ ID NO: 7]

ATGGATAAAATTGAAACCATACTTAAAAGTATTGAACCGATTATTATGAACTGTCGGAATAAACTAA
AATTCCTTCTCGGGAATTAGACGACTATATGCAGGAAGGGATGATTATTGCTTTAGAGATGTACCATC
AACTCTTATTAGATCCACCAGATGATGACTTAACTTCTATGTCTATTTCAAAGTCAGGTATTCTTGT
TTCTTAATTGATCACTATCGCAAAGCTATGGCAGTCAAGAGAAAATTCGACCAGCTTGACTATTGTGA

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ACTTTCTGAGTCTGTTAATCTTTTGTATCACAACAAAATGTGTCTGAAAACGTATGTATAACTTGT
TGTGTCAAGAAATACACTTGGTTTTATCCCCGAGGAGCTCAAGCTTTTGAGGCACTTATTGA

[SEQ ID NO: 8]

MDKIETILKSIEPIIMNCRKTKIPSWELDDYMQEGMIIALEMYHQLLLDPPDDDFNFYVYFKVRYSC
FLIDHYRKAMAVKRKFDQLDYCELSSESVNLPDQHKQNVSENVMYNLLCQEIHLVLSPEELKLFELI

[SEQ ID NO: 9]

ATGGATAGCATAGAAATGATGCTTCAAATATTGAGCCAATTATTATGAATTGTAGTAAACAACCTAG
GATTCCATCTTGGGAGCTAGATGATTACATGCAGGAGGGGATGATTATTGCACTGGAATGTATCAA
ATAGACATAACATCAATAACGGTAACGCGTTTAATTTCTATGCTATTTTAAAGTCAGGTATTCTGT
TACCTGATAGATAGTTTTAGAAAGGCTAACGCATATAAAAGAAAATTTGATCAACCATTATATTGTGA
AATATCTGAAGCCTTCAACCTTTATGATCACCACCAAAATGTTGCAGACAATGTCTGTTATCAGCTAT
TGCAAGTTGAAATCTTGAGATATTAACACCAGATGAAGCTGATTATTTATGACCTTGAAAAATGGT
GGGAAAGTAGAGAGAAATAAAAGTATAGATTAAAGAAAAAATTATTGATTATCTTAAAGACATGTT
ATGA

[SEQ ID NO: 10]

MDSIEMMLQNIPIIMNCSKTRIPSWELDDYMQEGMIIALEMYQNRHNINNGNAPNFYVYFKVRYSC
YLIDSFRKANAYKRKFDQPLYCEISEAFNLYDHHQNVADNVCYQLLQVEILEILTPDEADLFMTLKNG
GKVERNKKYRLKKKIIDYLDML

[SEQ ID NO: 11]

ATGGAGACTTTAGAAGCCATGCTCAAAACATTGAACCTATTATTATGAATTGTCAAAGATGGCAA
AATACCTTCCTGGGATATTGACGATTATATGCAGGAGGGGAGGATCATTGCATTAGACTTGATAATC
AGCTAGCAGAAAGAATGGAGACGGATGAGGTGAACCTTTACGCTCTACTTCAAAGTCAGATATACCTGT
TTCTTGATTGATACTTACCGTAAGACAAATGCCTTTAAAGAAAATTTGACCAACCGATTTACTTAGA
TGTATCCGAAGCATTTAATCTGTATGATCATAAGCAGAATGTCGCTGATAATGTCATGTATACTTTAT
TGCATCAGGAGATTCTAGACATCTTAACGCCTGTAGAAATTCAAACGCTAAACGCCTAAAAAGGGGA
GAAAAGGTCGACCGCAATAAAAAATTTAGGATTAAAAAGAAGATTATCAACTATATTAATCAGATTTT
CTAG

[SEQ ID NO: 12]

METLEAMLKNIPIIMNCQKMAKIPSWDIDDYMQEGRIIALDLYNQLAERMETDEVNFYVYFKVRYTC
FLIDTYRKTNAFKRKFDQPIYLDVSEAFNLYDHHQNVADNVMTLLHQEILDILTPVEIQTLNALKRG
EKVDRNKKFRIKKKIINYINQIF

[SEQ ID NO: 13]

ATGGAGCATAATTTAGATATGGAGCAGCTGGAAGAAATTTTCATTCTGTCCAACATATTGTGTGGAA
GAACAGTCGTTGATTCCGATAAATTTTGGACGTTTGATGACTATCAGCAGGAAGGGCGCTTGGTAT
TATACGATTTGCTGGGAGATGGTGTGACGCAAAGGAACCTATTTTGCATTTTAAGGTACGCTATAAG
CAGAGACTTATTGATATTAAGAAGGGAGCGGGCTTTTAAAGGGGTTTGGATTGCGGGACTGGCTT
AGATATATACGAATATTCTGATGCTCTAAAGGGGAAAGCAGCCAGTCCAGAACATATCCTGATTTCTG
GAAGTTTACTTGAAGAAGTTTTTGAAACTTAAATTTACGCTACCGACGGCTCCTCAAAGTTACCTC
GCCGCGCATGAATTGCACCGTATGGAAAAGTATCGTTGAAGGAAAAAATAACGAATATATTATATGA
ACAGCAGTGA

- continued

[SEQ ID NO: 14]

MEHNLDMEQLEEIFHSVQHIVWKNRLIPINFWTFDDYQQEGRVLVLYDLLGDGVTQRNLFCHFKVRYK
QRLIDIKRRERAFKRGFDCGTGLDIYEYSDALKGKAASPEHILISGSLLEEVFENLNLRYRRLKSYL
AGDELHRMEKYRLKEKITNILEYQQ

[SEQ ID NO: 15]

ATGGCAGAAAATAATTTAGATAAAGAACAGCTTGAAGAGTTATTCCATTCACCTCAACATATTGTTTG
GAAGAACAGTCATTTAATTAAATAAATTTTGGACAATGGATGATTATCAGCAAGAAGGGCGACTGG
TTTTATACCAGTTACTTGAAGATGGCGTGACACAGGAAAACTATTTGCCATTTTAAAGTGCATAT
AAGCAACGGTTGATTGATATAAAAAAGACGAGAAAGAGCATTTAAGCGGGTTTGTATTGTGGGGCTGG
TTTAGATATATATGAGTATTCTGATGCCCTGAAAGGCAAAGCTACCAGTCCTGAATATACTTAATTT
CAGTTACTTTACTTGAAGAGGTTTCATCAAAGTTTGTAGTTTGTAGATACCGCAATTTATTGGAGAATCAT
CTGTGAGGAGTGGAGTTGCATCGAATGGAAAAATACCGTTTAAAGGAAAAATCAAGAGAATACTCTA
TGAAGAAGAATGA

[SEQ ID NO: 16]

MAENLDKEQLEELFHSVQHIVWKNRLIPINFWTFDDYQQEGRVLVLYQLLEDGVTQEKLFCHFKVRY
KQRLIDIKRRERAFKRGFDCGAGLDIYEYSDALKGKATSPEYNLISVTLLEEVHQSLSLRNLLLENH
LSGVELHRMEKYRLKEKIKRILEYEE

[SEQ ID NO: 17]

ATGGAGCATAATTTAGATATGGAGCAGCTGGAAGAGATATTCATTCTGTTCAACATATTGTATGGAA
GAATAGTCGTTGATTCCGATAAATTTTGGACGATAGATGACTATCAGCAGGAAGGGCGTTTGGTAT
TATATGATTTACTTGAGGATGGTGTGACACAAAGAAAACTTTTGGCATTTTAAAGTACGTTATAAG
CAGAGACTTATTGATATTAAGAAGGGAGCGGGCTTTTAAAGGGGTTTGTACTGTGGACTGGGCT
AGATATTTACGAATATTGAGATGCTTTAAAGGAAAAGTAGCCAGTCCAGAACATACTCTGATTCTG
GCAGTTTGCTTGAAGAAGTTTGTAGAAACTTAAATTTACGCTACCGTGCTCTTCTTAAAGTTACCTT
GCTGGTGATGAAGTGCATCGAATGGAAAAACATCGTTTGAAGAAAAAATAATAAAATATTATATGA
TGAACAGTGA

[SEQ ID NO: 18]

MEHNLDMEQLEEIFHSVQHIVWKNRLIPINFWTFDDYQQEGRVLVLYDLLEDGVTQRNLFCHFKVRYK
QRLIDIKRRERAFKRGFDCGTGLDIYEYSDALKGKVASPEHTLISGSLLEEVLENLNLRYRALLKSYL
AGDELHRMEKHLKEKIKILEYEQ

[SEQ ID NO: 19]

TTGAAACCGATCGTTTCAAATCTATGAGAACATTAAAAATCAATTTTGGACTACAGAGGATTATCA
TCAAGAGGGTCTAATTACATTAAATGAAATATTAAATTCAGGATGTAAGGAGTCACAACTATACATTC
ACTTTAAAGTCAAATATCGACAAAAGCTAATAGACGTGATTAGAAAATCACAGGCGCAAAAAGAATC
TGGGATAATGCAGAGAGTATTGATGTTTACGAATCTGAAAATCAAATTAATTCAGTAACTCAAACCC
CGAAGACATAATAGTCTATGACAGTCTTGTAAGGAAGTAATAACAAAATTAACACCTTCATACCGGA
AACTACTGAAACGACATCTAAGAGGTGAGGATGTGACAAGGATGGAAAAATACAGACTGAAGGAACGA
ATCAAAACAAATTTTATTGATGGTGATTGA

[SEQ ID NO: 20]

MKPIVSKSMRTLKINFWTTEDYHQGLITLNEILNSGCKESQLYIHFKVKYRQKLIDVIRKSQAQKRI
WDNAESIDVYESENQINSNSNPEDIIIVYDSLVEKITKLTPSYRKLLKRHLRGEDVTRMEKYRLKER
IKQILFDGD

- continued

[SEQ ID NO: 21]
 ATGGATAAGATTGAAACCATACTTAAAAATATTGAACCGATTATCATGAAGTGTGAAAAAACTAA
 CATCCCTTCTCTGGCAATTAGACGACTATCTCCAGGAAGGCATGATTATTGCTCTAGAGATGTATCATC
 AACTTTTATTAGACCCACCAGATGATGACTTTAACTTCTATGTTTATTTCAAAGTGAGATATTCTTGT
 TTCTTGATTGATCAGTATCGGAGAAACATGGCTGTCAAAGAAAATTCGACCAGATTGACTATTGTGA
 ACTATCTGAGGCGTTTATCTTTTGTATCAAAATCAAGATGTCTCTGAAAACGTCATGTATAATTTGT
 TATGTCAAGAAATACACTTGCTTCTATCTCTGAAGAACGAGAGCTTTTGTAGGCACTTAAAAATGGA
 CAGAAGATTGACCGTAATCAAAGTTTCGTATCAAGAAGAAAATTATTGAATATATTAAGAGGTTTGTG
 GTGA

[SEQ ID NO: 22]
 MDKIETILKNIEPIIMNCRKKNIPSWQLDDYLQEGMIIALEMYHQLLLDPDDDFNFYVYFKVRYSC
 FLIDQYRRNMAVKRKFDQIDYCELSAFLFDQNDVSENVMYNLLCQEIHLLLSPEERELFEALKNG
 QKIDRNQKFRKKKIIIEYIKRFW

[0139] In some embodiments, said comX gene has the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or is a variant of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 encoding respectively the ComX protein of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 or is a variant of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 encoding respectively a functional ComX protein having at least 90% identity or at least 90% similarity to a ComX protein of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6. In a particular embodiment, said comX gene is used when the *Lactococcus* strain in step a) is a *Lactococcus lactis* strain.

[0140] In a particular embodiment, when the strain of step a) is a *Lactococcus lactis* subsp. *lactis* strain, the comX gene comprises the nucleotide sequence of SEQ ID NO:1, any sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to SEQ ID NO:1, a variant of SEQ ID NO:1 encoding the ComX protein of SEQ ID NO:2 or a variant of SEQ ID NO:1 encoding a functional ComX protein having at least 90% identity or at least 90% similarity to a ComX protein of SEQ ID NO:2.

[0141] In a particular embodiment, when the strain of step a) is a *Lactococcus lactis* subsp. *cremoris* strain, the comX gene comprises the nucleotide sequence of SEQ ID NO:3 or SEQ ID NO:5, any sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to SEQ ID NO:3 or SEQ ID NO:5 or a variant of SEQ ID NO:3 or SEQ ID NO:5 encoding respectively the ComX protein of SEQ ID NO:4 or SEQ ID NO:6 or a variant of SEQ ID NO:3 or SEQ ID NO:5 encoding respectively a functional ComX protein having at least 90% identity or at least 90% similarity to a ComX protein of SEQ ID NO:4 or SEQ ID NO:6.

[0142] In some embodiments, said comX gene has the nucleotide sequence of SEQ ID NO:7 or has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%

identity to the nucleotide sequence of SEQ ID NO:7 or is a variant of SEQ ID NO:7 encoding the ComX protein of SEQ ID NO:8, or is a variant of SEQ ID NO:7 encoding a functional ComX protein having at least 90% identity or at least 90% similarity to a ComX protein of SEQ ID NO:8. In a particular embodiment, said comX gene is used when the *Lactococcus* strain in step a) is a *Lactococcus raffinolactis* strain.

[0143] In some embodiments, said comX gene has the nucleotide sequence of SEQ ID NO:9 or has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the nucleotide sequence of SEQ ID NO:9 or is a variant of SEQ ID NO:9 encoding the ComX protein of SEQ ID NO:10, or is a variant of SEQ ID NO:9 encoding a functional ComX protein having at least 90% identity or at least 90% similarity to a ComX protein of SEQ ID NO:10. In a particular embodiment, said comX gene is used when the *Lactococcus* strain in step a) is a *Lactococcus plantarum* strain.

[0144] In some embodiments, said comX gene has the nucleotide sequence of SEQ ID NO:11 or has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the nucleotide sequence of SEQ ID NO:11 or is a variant of SEQ ID NO:11 encoding the ComX protein of SEQ ID NO:12, or is a variant of SEQ ID NO:11 encoding a functional ComX protein having at least 90% identity or at least 90% similarity to a ComX protein of SEQ ID NO:12. In a particular embodiment, said comX gene is used when the *Lactococcus* strain in step a) is a *Lactococcus piscium* strain.

[0145] In a particular embodiment, said comX gene has the nucleotide sequence of SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17, any sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17 or a variant of SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17 encoding respectively the ComX protein of SEQ ID NO:14 or SEQ ID NO:16 or SEQ ID NO:18 or a variant of SEQ ID NO:13 or SEQ ID NO:15 or SEQ ID NO:17 encoding respectively a functional ComX protein

having at least 90% identity or at least 90% similarity to a ComX protein of SEQ ID NO:14 or SEQ ID NO:16 or SEQ ID NO:18. In a particular embodiment, said comX gene is used when the *Lactococcus* strain in step a) is a *Lactococcus garvieae* strain.

[0146] In some embodiments, said comX gene has the nucleotide sequence of SEQ ID NO:19 or has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the nucleotide sequence of SEQ ID NO:19 or is a variant of SEQ ID NO:19 encoding the ComX protein of SEQ ID NO:20, or is a variant of SEQ ID NO:19 encoding a functional ComX protein having at least 90% identity or at least 90% similarity to a ComX protein of SEQ ID NO:20. In a particular embodiment, said comX gene is used when the *Lactococcus* strain in step a) is a *Lactococcus fujiensis* strain.

[0147] In some embodiments, said comX gene has the nucleotide sequence of SEQ ID NO:21 or has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least

95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the nucleotide sequence of SEQ ID NO:21 or is a variant of SEQ ID NO:21 encoding the ComX protein of SEQ ID NO:22, or is a variant of SEQ ID NO:21 encoding a functional ComX protein having at least 90% identity or at least 90% similarity to a ComX protein of SEQ ID NO:22. In a particular embodiment, said comX gene is used when the *Lactococcus* strain in step a) is a *Lactococcus chungangensis* strain.

[0148] By way of example and for the avoidance of doubt, in particular embodiments, where a comX gene is specified as having a particular nucleotide sequence, it is understood that the comX gene comprises said nucleotide sequence. In particular other embodiments, where a comX gene is specified as having a particular nucleotide sequence, it is understood that the comX gene consists of said nucleotide sequence.

[0149] In some embodiments, variants as defined herein of comX genes are selected from the list of DNA sequences disclosed in Table 1 below:

TABLE 1

Strain	Accession number	Position of comX, from start to stop
<i>Lactococcus lactis</i> A106	CP009472.1	From 2260881 to 2261372 (reverse)
<i>Lactococcus lactis</i> Bp11	JRFX01000055.1	From 34668 to 35159 (forward)
<i>Lactococcus lactis</i> L11596	LDEK01000015.1	From 33401 to 33892 (forward)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> A17	JQIC01000009.1	From 6445 to 6936 (reverse)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> A76	CP003132.1	From 2293232 to 2293723 (reverse)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> AM2	LITE01000081.1	From 9954 to 10444 (forward)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> B40	LITC01000320.1	From 10186 to 10677 (forward)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> DPC6856	LAVW01000168.1	From 445 to 936 (reverse)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> GE214	AZSI01000020.1	From 186 to 677 (reverse)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> HP	JAUH01000192.1	From 40 to 531 (reverse)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> IBB477	JMMZ01000035.1	From 92323 to 92814 (reverse)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> KW10	LIYF01000023.1	From 40421 to 40912 (forward)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> KW2	CP004884.1	From 2276371 to 2276862 (reverse)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> LMG6897	LISZ01000238.1	From 10034 to 10525 (forward)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> Mast36	JZUI01000076.1	From 310 to 801 (reverse)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> MG1363	AM406671.1	From 2376782 to 2377273 (reverse)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> NBRC 100676	BCVK01000073.1	From 9879 to 10370 (forward)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> NZ9000	CP002094.1	From 2377598 to 2378089 (reverse)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> SK11	CP000425.1	From 2283008 to 2283498 (reverse)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> TIFN1	ASXF01000005.1	From 5621 to 6112 (forward)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> TIFN3	ATBE01000400.1	From 431 to 922 (reverse)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> TIFN5	ATBC01000090.1	From 315 to 809 (reverse)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> TIFN6	ATBB01000278.1	From 265 to 756 (forward)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> TIFN7	ATBA01000081.1	From 5620 to 6111 (forward)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> UC509.9	CP003157.1	From 2107522 to 2108013 (reverse)
<i>Lactococcus lactis</i> subsp. <i>cremoris</i> V4	LIYG01000005.1	From 8625 to 9116 (forward)
<i>Lactococcus lactis</i> subsp. <i>hordniae</i> NBRC 100931	BCVL01000030.1	From 70 to 561 (reverse)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> 1AA59	AZQT01000035.1	From 118 to 609 (reverse)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> 511	JNLP01000001.1	From 1703029 to 1703520 (reverse)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> A12	LT599049.1	From 2415707 to 2416198 (reverse)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> ATCC 19435	LKLC01000004.1	From 32310 to 32801 (forward)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> bv. <i>diacetylactis</i> DRA4	LIWD01000119.1	From 147 to 638 (reverse)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> CV56	CP002365.1	From 2213300 to 2213791 (reverse)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> DPC6853	LAVD01000101.1	From 544 to 1035 (reverse)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> E34	LKLD01000014.1	From 197 to 688 (reverse)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> I11403	AE005176.1	From 2223528 to 2224019 (reverse)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> IO-1 DNA	AP012281.1	From 2287126 to 2287617 (reverse)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> JCM 7638	BBAP01000017.1	From 34164 to 34656 (forward)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> K231	LKLE01000041.1	From 32159 to 32650 (forward)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> K337	LKLF01000041.1	From 34909 to 35400 (forward)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> KF134	LKLJ01000010.1	From 34939 to 35430 (forward)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> KF147	CP001834.1	From 2446402 to 2446893 (reverse)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> KF201	LKLM01000024.1	From 28747 to 29238 (forward)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> KF24	LKLH01000011.1	From 34116 to 34607 (forward)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> KF282	LKLN01000033.1	From 170 to 661 (reverse)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> KLDS 4.0325	CP006766.1	From 2407603 to 2408094 (reverse)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> LMG 7760	JQCM01000018.1	From 37736 to 38227 (forward)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> LMG8526	LKLQ01000046.1	From 38499 to 38993 (forward)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> NCDO 2118	CP009054.1	From 2402923 to 2403414 (reverse)

TABLE 1-continued

Strain	Accession number	Position of comX, from start to stop
<i>Lactococcus lactis</i> subsp. <i>lactis</i> S0	CP010050.1	From 2359456 to 2359947 (reverse)
<i>Lactococcus lactis</i> subsp. <i>lactis</i> UC317	LKLY01000004.1	From 36130 to 36621 (forward)
<i>Lactococcus lactis</i> WG2	LXWJ01000007.1	From 37921 to 38412 (forward)
<i>Lactococcus raffinolactis</i> NBRC 100932	BCVN01000102.1	From 139 to 617 (forward)
<i>Lactococcus piscium</i> CNCM 1-4031	FLZT01000001.1	From 149 to 628 (forward)
<i>Lactococcus piscium</i> MKFS47	LN774769.1	From 1708720 to 1709199 (forward)
<i>Lactococcus garvieae</i> 122061	AP017373.1	From 1356405 to 1356890 (forward)
<i>Lactococcus garvieae</i> 8831	AFCD01000005.1	From 510 to 995 (forward)
<i>Lactococcus garvieae</i> Lg-ilsanpaik-gs201105	JPUJ01000002.1	From 180817 to 181302 (reverse)
<i>Lactococcus garvieae</i> LG9	AGQY01000137.1	From 5631 to 6116 (reverse)
<i>Lactococcus garvieae</i> M79	FOTJ01000023.1	From 3224 to 3709 (forward)
<i>Lactococcus garvieae</i> NBRC 100934	BBJW01000010.1	From 105946 to 106431 (reverse)
<i>Lactococcus garvieae</i> PAQ102015-99	LXWL01000009.1	From 238437 to 238922 (reverse)
<i>Lactococcus garvieae</i> TB25	AGQX01000090.1	From 28088 to 28573 (reverse)
<i>Lactococcus garvieae</i> TRF1	AVFE01000015.1	From 42141 to 42626 (reverse)

[0150] As used herein a comX gene is understood to be a gene that encodes a functional ComX protein in the strain where it is expressed. By “functional ComX protein” it is meant a protein which induces or is able to induce the expression of genes regulated by the Com-box, and at least one of the late competence genes selected from comFA, comFA, comGA, dprA, coiA, ssbA, radA, radC, recA, and recX.

[0151] The ComX protein may have the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20 or SEQ ID NO:22, or an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20 or SEQ ID NO:22, or an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% similarity to the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20 or SEQ ID NO:22.

[0152] In some embodiments, the ComX protein may have the amino acid sequence of SEQ ID NO:2, or an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence of SEQ ID NO:2 or an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% similarity to the amino acid sequence of SEQ ID NO:2. In a particular embodiment, said ComX protein is used when the *Lactococcus* strain in step a) is a *Lactococcus lactis* subsp. *lactis* strain.

[0153] In some embodiments, the ComX protein may have the amino acid sequence of SEQ ID NO:4 or SEQ ID NO:6, or an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence of SEQ ID NO:4 or SEQ ID NO:6 or an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least

96%, at least 97%, at least 98%, or at least 99% similarity to the amino acid sequence of SEQ ID NO:4 or SEQ ID NO:6. In a particular embodiment, said ComX protein is used when the *Lactococcus* strain in step a) is a *Lactococcus lactis* subsp. *cremoris* strain.

[0154] In some embodiments, the ComX protein may have the amino acid sequence of SEQ ID NO:8, or an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence of SEQ ID NO:8 or an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% similarity to the amino acid sequence of SEQ ID NO:8. In a particular embodiment, said ComX protein is used when the *Lactococcus* strain in step a) is a *Lactococcus raffinolactis* strain.

[0155] In some embodiments, the ComX protein may have the amino acid sequence of SEQ ID NO:10, or an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence of SEQ ID NO:10 or an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% similarity to the amino acid sequence of SEQ ID NO:10. In a particular embodiment, said ComX protein is used when the *Lactococcus* strain in step a) is a *Lactococcus plantarum* strain.

[0156] In some embodiments, the ComX protein may have the amino acid sequence of SEQ ID NO:12, or an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence of SEQ ID NO:12 or an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% similarity to the amino acid sequence of SEQ ID NO:12. In a particular embodiment, said ComX protein is used when the *Lactococcus* strain in step a) is a *Lactococcus piscium* strain.

[0157] In some embodiments, the ComX protein may have the amino acid sequence of SEQ ID NO:14, SEQ ID NO:16 or SEQ ID NO:18, or an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at

least 99% identity to the amino acid sequence of SEQ ID NO:14, SEQ ID NO:16 or SEQ ID NO:18 or an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% similarity to the amino acid sequence of SEQ ID NO:14, SEQ ID NO:16 or SEQ ID NO:18. In a particular embodiment, said ComX protein is used when the *Lactococcus* strain in step a) is a *Lactococcus garvieae* strain.

[0158] In some embodiments, the ComX protein may have the amino acid sequence of SEQ ID NO:20, or an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence of SEQ ID NO:20 or an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% similarity to the amino acid sequence of SEQ ID NO:20. In a particular embodiment, said ComX protein is used when the *Lactococcus* strain in step a) is a *Lactococcus fujiensis* strain.

[0159] In some embodiments, the ComX protein may have the amino acid sequence of SEQ ID NO:22, or an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the amino acid sequence of SEQ ID NO:22 or an amino acid sequence having at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% similarity to the amino acid sequence of SEQ ID NO:22. In a particular embodiment, said ComX protein is used when the *Lactococcus* strain in step a) is a *Lactococcus chungangensis* strain.

[0160] According to the invention, when a ComX protein is defined by its amino acid sequence having a percentage of identity or percentage of similarity to a specific SEQ ID, said ComX protein is a functional ComX protein as defined herein.

[0161] By way of example and for the avoidance of doubt, in particular embodiments, where a ComX protein is specified as having a particular amino acid sequence, it is understood that the ComX protein comprises said amino acid sequence. In particular other embodiments, where a ComX protein is specified as having a particular amino acid sequence, it is understood that the ComX protein consists of said amino acid sequence.

[0162] In some embodiments, ComX proteins having percentage of identity or percentage of similarity as defined herein are selected from the list of protein sequences derived, after translation, from the list of DNA sequences disclosed in Table 1 above.

[0163] Preferably, reference to a sequence which has a percentage identity or similarity to any one of the SEQ ID NOs detailed herein refers to a sequence which has the stated percent identity or similarity with the SEQ ID NO referred to, over the entire length of the two sequences. Percentage (%) sequence identity is defined as the percentage of amino acids or nucleotides in a candidate sequence that are identical to the amino acids or nucleotides in a reference sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Percentage (%) sequence similarity is defined as the percentage of amino acids in a candidate sequence that are similar to the amino acids in a reference sequence, after

aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence similarity. Similarity between amino acids is based on established amino acid substitution matrices such as the PAM series (Point Accepted Mutation; e.g. PAM30, PAM70, and PAM250) or the BLOSUM series (BLOck SUBstitution Matrix; e.g. BLOSUM45, BLOSUM50, BLOSUM62, BLOSUM80, and BLOSUM90). Alignment for purposes of determining percent sequence identity or similarity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as CLUSTALW, CLUSTALX, CLUSTAL Omega, BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. In a particular embodiment, similarity between amino acids is determined using the BLASTp software with the BLOSUM62 matrix. Appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared, or gap penalties to be introduced, can be determined by known methods.

[0164] In a particular embodiment, when the modulation in step b) results from an exogenous comX gene, said exogenous comX gene is (obtained) from a strain of the same species, in particular of the same subspecies, as the strain provided in step a). In any case, the exogenous comX gene needs to be functional, in particular needs to encode a functional ComX protein, as defined herein in the strain provided in step a).

Exogenous DNA Polynucleotide

[0165] The method of the present invention comprises the step of contacting the strain of step b) with an exogenous DNA polynucleotide in a medium and incubating the resulting mixture for integration of the exogenous DNA into the genome of said strain [step c].

[0166] As used herein the term “exogenous DNA polynucleotide” refers to a DNA polynucleotide that is brought into the cytoplasm of said strain, in order to be integrated into the genome of said strain (target sequence).

[0167] In a particular embodiment, the method comprises carrying out step (b) [ComX modulation] and then carrying out step (c) [contact with the exogenous DNA polynucleotide] [i.e., that step (c) is carried out on a strain obtained following step (b)]. Thus, the method comprising the steps of:

[0168] (a) providing a strain of the *Lactococcus* genus, wherein said strain is transformable through natural competence;

[0169] (b) modulating the production of a ComX protein in said strain;

[0170] (c) contacting said strain obtained in step (b) with an exogenous DNA polynucleotide in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and

[0171] (d) selecting a strain which has integrated the exogenous DNA polynucleotide into its genome.

[0172] In another embodiment, the method comprises carrying out simultaneously step (b) [ComX modulation] and step (c) [contact with the exogenous DNA polynucleotide]. This option is appropriate when the ComX modulation is the result of the expression of the endogenous comX

gene or of the increase of the expression of the endogenous comX gene of said strain. Thus, the method comprising the steps of:

- [0173] (a) providing a strain of the *Lactococcus* genus, wherein said strain is transformable through natural competence;
- [0174] (b) modulating the production of a ComX protein in said strain;
- [0175] (c) contacting said strain with an exogenous DNA polynucleotide in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and
- [0176] (d) selecting a strain which has integrated the exogenous DNA polynucleotide into its genome;
- [0177] wherein step (b) and step (c) are carried out simultaneously.
- [0178] In a particular embodiment, the sequence of the exogenous DNA polynucleotide used in step c) share some similarities or identities with the genome of the *Lactococcus* strain to be transformed (of step a). In a particular embodiment, the exogenous DNA polynucleotide used in step c) is designed such that its 5' part and its 3' part are identical or highly similar to parts of the genome of the *Lactococcus* strain to be transformed (of step a), while its central part can be different from the genome of the *Lactococcus* strain to be transformed (of step a). The high similarity of the arms with the regions surrounding the target sequence can be determined by the person skilled in the art using common general knowledge, in particular by reference to homologous recombination.
- [0179] Thus, to replace a target sequence by a mutated sequence or a truncated sequence or a supplementary sequence in the genome of the *Lactococcus* strain to be transformed (of step a), the exogenous DNA polynucleotide used in step c) is designed such that:
 - [0180] its 5' part is identical or highly similar to the region of the genome of the *Lactococcus* strain to be transformed which is on one side of the target sequence;
 - [0181] its central part contains the replacing sequence (i.e., the mutated sequence or the truncated sequence or the supplementary sequence); and
 - [0182] its 3' part is identical or highly similar to the region of the genome of the *Lactococcus* strain to be transformed which is on the other side of the target sequence.
- [0183] The 5' part and 3' part are long enough to ensure efficient recombination. In a particular embodiment, each of the 5' part and 3' part is from 0.5 to 5 kb in length. The size of the arms can be determined by the person skilled in the art using common general knowledge, in particular by reference to homologous recombination.
- [0184] In a particular embodiment, the exogenous DNA polynucleotide used in step c) is (obtained) from a strain of the *Lactococcus* genus.
- [0185] In a particular embodiment, said exogenous DNA polynucleotide used in step (c) is (obtained) from a strain of the same species, in particular of the same subspecies, as the strain provided in step (a).
- [0186] In a particular embodiment, the exogenous DNA polynucleotide used in step c) is from a strain of the *Lactococcus lactis* species. In a particular embodiment, the exogenous DNA polynucleotide used in step c) is from a strain of the same *Lactococcus lactis* subspecies as the strain

provided in step a). In a particular embodiment, the exogenous DNA polynucleotide used in step c) is from a strain of a *Lactococcus lactis* subspecies which is different from the strain provided in step a).

[0187] In a particular embodiment, the exogenous DNA polynucleotide used in step c) is from a strain of the *Lactococcus raffinolactis* species

[0188] The exogenous DNA polynucleotide may encode part of a gene sequence, a gene sequence, or a plurality of gene sequences. The gene sequence may be operably linked to transcription regulator(s). In a particular embodiment, the exogenous DNA polynucleotide is linear. The exogenous DNA polynucleotide may be designed to facilitate its incorporation within the genome of the *L. lactis* strain by homologous recombination (e.g. the exogenous DNA polynucleotide may comprise one or more recombination arms). The exogenous DNA polynucleotide may be a single stranded linear DNA.

[0189] The exogenous DNA polynucleotide, when incorporated into the genome of said *Lactococcus* strain leads to genetic modification of the strain such as gene replacement (to add or to remove a mutation), gene addition (to add a new gene or to duplicate an existing gene), gene deletion (to remove part or the totality of a gene), modification of non-coding region (to modulate expression of a gene). Typically, the exogenous DNA polynucleotide, when incorporated into the genome of said *Lactococcus* strain confers an interesting or useful phenotype, e.g. modified kinetic of acidification, improved resistance to bacteriophage, modified capability to grow in milk, modified texturing properties, improved safety of the strain. For example, improved bacteriophage resistance could be achieved by incorporating genes coding for a restriction/modification system into the strain genome or by introducing a mutation or a deletion into the *pip* gene.

[0190] As an example, growth of a *L. lactis* strain in milk could be improved by inserting into the chromosome the *prtP* and *prtM* genes that allow casein hydrolysis and better nitrogen nutrition; alternatively, these genes could be inactivated to reduced milk proteolysis in cheese. *hisDC* and *tyrDC* are genes known to be responsible for biogenic amine production (histamine and tyramine, respectively) in a diversity of lactic acid bacteria; disruption or mutation of these genes could help to prevent safety issues related to cheese consumption.

[0191] In a particular embodiment, the exogenous DNA polynucleotide has a minimal size selected from the group consisting of 100 bp, 200 bp, 500 bp, 1 kb, 2 kb and 5 kb, and a maximal size selected from the group consisting of 500 bp, 1 kb, 2 kb, 5 kb, 10 kb, 20 kb and 50 kb. In a particular embodiment, the size of the exogenous DNA polynucleotide may be between 100 bp and 50 kb, more preferably between 500 bp to 20 kb, even more preferably between 1 kb to 10 kb.

[0192] The concentration of exogenous DNA polynucleotide in the medium of step (c) may be between 0.5 mg/L and 1 g/L, preferably between 1 mg/L and 500 mg/L, more preferably between 5 mg/L and 100 mg/L, even more preferably between 10 mg/L and 50 mg/L of medium.

Selection of Transformed Strains

[0193] The method of the present invention comprises the step of selecting a strain which has integrated the exogenous DNA polynucleotide into its genome [step d)].

[0194] If needed, selection is carried out on some cells of colonies that have been previously obtained by multiplying, in the appropriate medium, cells obtained at the end of step c) (or at the end of the simultaneous steps b) and c), when appropriate).

[0195] Various methods for the selection of transformed bacteria are well known in the art (see, e.g. Sambrook et al.) and may be routinely applied by the person skilled in the art, such as PCR, DNA sequencing

[0196] For example, when the exogenous DNA polynucleotide used in step c) provides a particular phenotype that the *Lactococcus* strain of step a) does not display (either a new phenotype or restoring a lost phenotype), it is possible to select strains which have integrated the exogenous DNA polynucleotide into their genome by selecting strains expressing the phenotype. This is the case for a strain having integrated in its genome an exogenous DNA polynucleotide mutated for the *pip* gene (that provides resistance to some bacteriophages).

[0197] For example, when the exogenous DNA polynucleotide used in step c) leads once integrated to a loss of a phenotype initially displayed by the *Lactococcus* strain of step a), it is possible to select strains which have integrated the exogenous DNA polynucleotide into their genome by selecting strains which do not display the phenotype any more. This is the case for an exogenous DNA polynucleotide bearing a mutated *hisDC* or *tyrDC* gene, which suppresses or decreases the production of histamine or tyramine, respectively.

[0198] As a particular example, the exogenous DNA polynucleotide may bear an antibiotic resistance gene. Accordingly, a *Lactococcus* strain which has integrated the exogenous DNA polynucleotide into its genome may be selected by plating onto a medium comprising said antibiotic. Only strains that express the appropriate antibiotic resistance gene, as a result of a successful transformation with the exogenous DNA polynucleotide, will multiply.

Growth Conditions

[0199] As described in Example 3, a positive effect on natural competence induction in *L. lactis* strains was observed when cells were pre-cultured in a complex medium before transferring the cells to a chemically defined medium (FIG. 3).

[0200] Accordingly, in some embodiments the medium of step (c) is a chemically defined medium. As used herein, the term “chemically defined medium” (CDM) refers to a medium for which the exact chemical composition is known. Preferably, the CDM may have the composition of the CDM set out in Sissler et al. (1999, Proc Natl Acad Sci USA 96:8985-8990). Thus, in an embodiment, the chemically defined medium (CDM) comprises 0.5 g/L NH_4Cl , 9.0 g/L KH_2PO_4 , 7.5 g/L K_2HPO_4 , 0.2 g/L MgCl_2 , 5 mg/L FeCl_2 , 50 mg/L CaCl_2 , 5 mg/L ZnSO_4 , 2.5 mg/L CoCl_2 , 0.05 g/L tyrosine, 0.1 g/L asparagine, 0.1 g/L cysteine, 0.1 g/L glutamine, 0.1 g/L isoleucine, 0.1 g/L leucine, 0.1 g/L methionine, 0.1 g/L tryptophan, 0.1 g/L valine, 0.1 g/L histidine, 0.2 g/L arginine, 0.2 g/L glycine, 0.2 g/L lysine, 0.2 g/L phenylalanine, 0.2 g/L threonine, 0.3 g/L alanine, 0.3 g/L proline, 0.3 g/L serine, 10 mg/L paraaminobenzoic acid, 10 mg/L biotin, 1 mg/L folic acid, 1 mg/L nicotinic acid, 1 mg/L pantothenic acid, 1 mg/L riboflavin, 1 mg/L thiamine, 2 mg/L pyridoxine, 1 mg/L cyanocobalamin, 5 mg/L orotic acid, 5 mg/L 2-deoxythymidine, 5 mg/L inosine, 2.5 mg/L

dl-6,8-thioctic acid, 5 mg/L pyridoxamine, 10 mg/L adenine, 10 mg/L guanine, 10 mg/L uracil, 10 mg/L xanthine, and 5 g/L glucose..

[0201] In some embodiments, prior to step (c) said strain is incubated in a pre-culture medium, preferably wherein the pre-culture medium is a complex medium, more preferably wherein the pre-culture medium is M17G (i.e., the M17 medium supplemented with glucose) or THBG (i.e., the THB medium supplemented with glucose).

[0202] The complex medium may be Todd Hewitt broth (THB) (Todd and Hewitt, 1932; Updyke and Nickle, 1954) or M17 broth (Terzaghi and Sandine, 1975). THB may comprise 500 g/L beef heart infusion, 20 g/L peptic digest of animal tissue, 2 g/L dextrose, 2 g/L sodium chloride, 0.4 g/L sodium phosphate, 2.5 g/L sodium carbonate. M17 broth may comprise: 0.5 g/L ascorbic acid, 5 g/L lactose, 0.25 g/L magnesium sulfate, 5 g/L meat extract, 2.5 g/L meat peptone (peptic), 19 g/L sodium glycerophosphate, 5 g/L soya peptone (papainic), 2.5 g/L tryptone, 2.5 g/L yeast extract.

Method for Identifying Strains Transformable by Natural Competence

[0203] In another aspect, the present invention relates to a method for identifying a strain of the *Lactococcus* genus which is transformable through natural competence. Said method comprises the following steps:

[0204] (a) providing a strain of the *Lactococcus* genus;

[0205] (b) transforming said strain with a plasmid expressing a *comX* gene having at least 90% identity, preferably having 100% identity, to the endogenous *comX* gene of said strain;

[0206] (c) contacting said strain obtained in step (b) with an exogenous marker DNA polynucleotide in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and

[0207] (d) determining the rate of recombination events;

[0208] wherein a rate of at least 1×10^{-6} transformants per μg of DNA is indicative of a strain which is transformable through natural competence.

[0209] The term “rate of recombination events” may be used interchangeably with the term “transformation rate”. The rate of recombination events is calculated by determining the ratio of the number of cells having integrated the exogenous marker DNA polynucleotide over the total number of viable cells. A rate of at least 10^{-6} was selected as a threshold, based on the observation that the level of spontaneous mutation in lactococci is less than 10^{-6} , typically around 10^{-7} mutants per μg of DNA [spontaneous means with no *comX* expression or overexpression].

[0210] By “at least 90% identity to the endogenous *comX* gene of said strain”, it is meant—as particular embodiments of the method—at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity. In a particular embodiment, said *comX* gene has 100% identity to the endogenous *comX* gene of said strain.

[0211] In a particular embodiment, said method is implemented with a strain of the *Lactococcus* genus selected from the group consisting of *Lactococcus lactis*, *Lactococcus raffinolactis*, *Lactococcus plantarum*, *Lactococcus piscium*, *Lactococcus garvieae*, *Lactococcus fujiensis* and *Lactococcus chungangensis*.

[0212] In a particular embodiment, said method is implemented with a strain of the *Lactococcus lactis* species. Said method comprises the following steps:

[0213] (a) providing a strain of the *Lactococcus lactis* species;

[0214] (b) transforming said strain with a plasmid expressing a comX gene having at least 90% identity to the polynucleotide sequence of SEQ ID NO:1, 3 or 5;

[0215] (c) contacting said strain obtained in step (b) with an exogenous marker DNA polynucleotide in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and

[0216] (d) determining the rate of recombination events;

[0217] wherein a rate of at least 1×10^{-6} transformants per μg of DNA is indicative of a strain of the *Lactococcus lactis* species which is transformable through natural competence.

[0218] In a particular embodiment, said method is implemented with a strain of the *Lactococcus raffinolactis* species. Said method comprises the following steps:

[0219] (a) providing a strain of the *Lactococcus raffinolactis* species;

[0220] (b) transforming said strain with a plasmid expressing a comX gene having at least 90% identity to the polynucleotide sequence of SEQ ID NO:7;

[0221] (c) contacting said strain obtained in step (b) with an exogenous marker DNA polynucleotide in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and

[0222] (d) determining the rate of recombination events;

[0223] wherein a rate of at least 1×10^{-6} transformants per μg of DNA is indicative of a strain of the *Lactococcus lactis* species which is transformable through natural competence.

[0224] In some embodiments, the comX gene is from a strain of the same species, in particular of the same subspecies, as the strain provided in step a). In some embodiments, the comX gene is identical (100% identity) to the polynucleotide sequence of the endogenous comX gene of the strain of step a).

[0225] In some embodiments, when the strain of step a) is a *Lactococcus lactis* subsp. *lactis* strain, the comX gene has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to the polynucleotide sequence of SEQ ID NO:1.

[0226] In some embodiments, when the strain of step a) is a *Lactococcus lactis* subsp. *cremoris* strain the comX gene has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to the polynucleotide sequence of SEQ ID NO:3 or SEQ ID NO:5.

[0227] In some embodiments, when the strain of step a) is a *Lactococcus raffinolactis* strain the comX gene has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to the polynucleotide sequence of SEQ ID NO:7.

[0228] In some embodiments, when the strain of step a) is a *Lactococcus plantarum* strain the comX gene has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%,

at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to the polynucleotide sequence of SEQ ID NO:9.

[0229] In some embodiments, when the strain of step a) is a *Lactococcus piscium* strain the comX gene has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to the polynucleotide sequence of SEQ ID NO:11.

[0230] In some embodiments, when the strain of step a) is a *Lactococcus garvieae* strain the comX gene has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to the polynucleotide sequence of SEQ ID NO:13, SEQ ID NO:15, or SEQ ID NO:17.

[0231] In some embodiments, when the strain of step a) is a *Lactococcus fujiensis* strain the comX gene has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to the polynucleotide sequence of SEQ ID NO:19.

[0232] In some embodiments, when the strain of step a) is a *Lactococcus chungangensis* strain the comX gene has at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to the polynucleotide sequence of SEQ ID NO:21.

[0233] It is preferable to use, as an exogenous marker DNA polynucleotide, a polynucleotide bearing a gene which is initially not present in the *Lactococcus* strain of step a) [even as a mutated version]. This would avoid that during step c) the *Lactococcus* strain acquires a functional gene by other means than natural competence, e.g. by spontaneous mutation of its genome.

[0234] As an example, the exogenous marker DNA polynucleotide bears a gene encoding a luciferase gene. Accordingly, a *Lactococcus* strain which has integrated the exogenous DNA polynucleotide into its genome may be selected for expression of the luciferase. Only strains that express the luciferase gene (i.e., integrated) will be detectable by bioluminescence.

[0235] As another example, the exogenous marker DNA polynucleotide bears an antibiotic resistance gene. Accordingly, a *Lactococcus* strain which has integrated the exogenous DNA polynucleotide into its genome may be selected by plating the cells onto a medium comprising said antibiotic.

[0236] An example of a method for identifying a strain of the *Lactococcus* genus which is transformable through natural competence according to the present invention (Assay A) may be performed using the following steps:

[0237] i) Providing a strain of the *Lactococcus* genus, in particular of the *Lactococcus lactis* species.

[0238] ii) Transforming said strain with a plasmid expressing a comX gene having at least 90% identity, preferably having 100% identity to the endogenous comX gene of said strain (e.g. the pGhP32comX_{MG} plasmid of Materials and Methods).

[0239] iii) Pre-culturing the transformed strain overnight in a complex medium supplemented with glucose (e.g. M17G) at 30° C.

[0240] iv) Diluting about 1.5 mL of the pre-culture in about 8.5 mL of fresh medium.

- [0241] v) After about 2 hours further growth at 30° C., washing the cells twice with distilled water and adjusting the OD₆₀₀ to 0.05 in a chemically defined medium (e.g. CDM) containing 5 µg mL⁻¹ erythromycin and an osmo-stabilizer (e.g. 5% [v/v] glycerol or 5% [w/v] mannitol).
- [0242] vi) Adding 5 µg of exogenous DNA polynucleotide bearing an antibiotic resistance gene to 300 µl of the culture medium (e.g. the 3.7 kb PCR product generated from the pGEMrpsL plasmid as described in Materials and Methods).
- [0243] vii) Incubating the resulting culture for about 6 hours at 30° C.
- [0244] viii) Plating the cells onto agar plates comprising the complex medium supplemented with glucose (e.g. M17G) and appropriate antibiotic (i.e. corresponding to the antibiotic resistance gene of the exogenous DNA polynucleotide) and incubating for about 48 hours.
- [0245] ix) Counting the colony forming units (CFU) and determining the transformation rate, wherein a transformation rate of at least 1×10⁻⁶ transformants per µg of DNA is indicative of a strain which is transformable through natural competence.
- [0246] The transformation rate may be calculated as the number of antibiotic-resistance CFU mL⁻¹ divided by the total number of viable CFU mL⁻¹.
- [0247] Various preferred features and embodiments of the present invention will now be described by way of non-limiting examples.

EXAMPLES

Example 1: Induction of the comGA Promoter by Constitutive comX Expression in Various Strains of the *Lactococcus* Species

- [0248] a) In *Lactococcus lactis* Subsp. *Cremoris* Strains (MG1363 and KW2)
- [0249] To test the ability of ComX to induce the late competence genes in *Lactococcus lactis* subsp. *cremoris* strains, a constitutive comX expression plasmid (pGhP32comX_{MG}) was created by cloning the comX gene from strain MG1363, under the control of the lactococcal P₃₂ promoter on the thermosensitive plasmid pG⁺host9. The latter was introduced in strain KW2 that contains a chromosomally-encoded P_{comGA[MG]}-luxAB transcriptional fusion (BLD101). The promoter of the late competence gene comGA (P_{comGA}) contains a putative ComX-binding motif and is used here as proxy for competence activation in the ComX strain.
- [0250] As alternative for more resistant strains to electrotransformation, and subsequently to chromosome integration, a portable luminescent reporter system was also constructed. This replicative plasmid carries the luminescent reporter P_{comGA[MG]}-lux with the P₃₂-comX_{MG} cassette. The pGhP32comX_{MG}-P_{comGA[MG]}-lux plasmid was transformed in strain MG1363. Specific P_{comGA[MG]}-lux/luxAB activities were monitored for the different strains constructed. The luminescent assays were performed in rich (M17G) and/or CDM media comparing the luciferase activity between the overexpressing comX strain and its related negative control (no additional comX copy).
- [0251] In the KW2 strain containing the P_{comGA[MG]}-luxAB reporter as mono-copy in their chromosome, specific luciferase activity was observed for KW2 containing the

P₃₂-comX cassette allowing the constitutive production of ComX. This confirms that comX expression can be carried out in various *L. lactis* subsp. *cremoris* strains using an exogenous comX gene obtained from the same strain or from a strain of the same subspecies. Eight recombinant clones of the KW2 ComX⁺ reporter strain were randomly selected and their specific luciferase activity was monitored in CDM growth conditions. This medium was chosen because it was shown to be permissive for competence development in various streptococcal species. To ensure reproducibility of the assay, exponentially-growing cells in complex medium (M17 conditions) were washed and inoculated in fresh CDM before starting the experiment. As expected, all tested ComX⁺ clones (cl01 to cl08) displayed between 10¹- and 10⁴-fold higher specific luciferase (Lux) activity than the control strain carrying the empty vector (FIGS. 2A and 2B).

[0252] Similar results were obtained with the portable luminescent reporter systems in MG1363 (FIG. 2C).

[0253] These results strongly suggest that, in the *L. lactis* subsp. *cremoris* strains MG1363 and KW2, ComX induces the comG operon. Additionally, these observations validate these reporter fusions (both chromosomal and plasmid-borne) as a tool to identify conditions capable to activate the comG-operon which is essential to natural transformation.

[0254] b) In a *L. lactis* Subsp. *Lactis* Strain (IL1403)

[0255] A constitutive comX expression plasmid (pGhP32comX_{IO}) was created by cloning the comX gene from strain IO-1, under the control of the lactococcal P₃₂ promoter on the thermosensitive plasmid pG⁺host9. A portable luminescent reporter system was also constructed; this replicative plasmid carries the luminescent reporter P_{comGA[IO]}-lux with the P₃₂-comX_{IO} cassette. The promoter of the late competence gene comGA (P_{comGA}) contains a putative ComX-binding motif and is used here as proxy for competence activation in the ComX⁺ strain.

[0256] This replicative plasmid pGhP32comX_{IO}-P_{comGA[IO]}-lux was transformed in strain IL1403 and specific P_{comGA[IO]}-lux activities were monitored. One of the IL1403 transformants produced specific P_{comGA[IO]}-lux activities confirming that ComX induces the comG operon (FIG. 2D).

Example 2: Analysis of Essential Late Corn Genes Present in *L. lactis* Genomes

[0257] Among *L. lactis* strains, genomic variability was previously investigated for comX and dprA alleles (Wydaue et al., 2006). While all strains (31/31) display a complete version of comX, the dprA content is variable among subspecies: 50% of the *lactis* strains (10/20) contain non-sense mutations in dprA while all *cremoris* strains (11/11) harbor an intact and potentially functional dprA gene.

[0258] Since dprA is hypothesized to be important in the natural competence mechanism, its integrity in *L. lactis* strains prompted us to further analyze the minimal set of late corn genes (17 candidate genes including comX; FIG. 1) in the genomes of 3 subsp. *cremoris* strains and 1 subsp. *lactis* strain which are publicly available (strains MG1363, SK11, KW2 and IL1403). This in silico analysis reveals that the genome of SK11 contains a high number of pseudogenes in key competence genes (between 5 and 8 incomplete late corn genes) due to transposon insertion or frameshifting events (nucleotide(s) insertion or deletion). In particular, the presence of transposable elements in comGA and/or comEC genes, which are respectively essential for pilus assembly

and DNA transport, strongly suggests that natural transformation is no more functional in those strains. Although the set of full-length competence genes in the laboratory strain MG1363 is larger, mutations in *comEC* (nucleotide insertion) and *coiA* (nonsense mutation) probably impair its ability to transform DNA by competence (Wegmann et al., 2007). Those mutations were also found in the genome of its isogenic derivative NZ9000, which strongly suggests that they do not result from DNA sequencing errors. As far as the *L. lactis* subsp. *lactis* IL1403 strain is concerned, its *dprA* gene contains nonsense mutations probably impairing its ability to transform DNA by competence. In contrast, strain KW2 of plant origin (corn fermentation) contains the whole set of known essential late genes required to fulfil natural DNA transformation, making it the best candidate to further study the functionality of competence in the *cremoris* subspecies. Two other strains from our collection, *L. lactis* subsp. *lactis* SL12651 and SL12653, were also found to contain the whole set of known essential late genes (FIG. 1).

Example 3: Effect of Growth Conditions on ComX Activation

[0259] We investigated the effect of pre-culturing and culturing conditions (M17G, THBG, and CDM) on the activation of the reporter fusion in the ComX⁺ strain. For this purpose, clone 02 (FIG. 2A) was selected since it exhibits the strongest Lux activity. Interestingly, more than a 20-fold variation in the maximum Lux activity was dependent on the pre- and culturing medium which was used (FIG. 3). Particularly, a positive impact of the transition of pre-culture cells from a complex medium to a defined medium was observed. The highest specific Lux activity ($\sim 3 \times 10^6$ RLU OD₆₀₀⁻¹) was obtained for a switch from M17G to CDM, followed by THBG to CDM, while all other combinations gave lower activities (between $\sim 1.5 \times$ and 5.5×10^5 RLU OD₆₀₀⁻¹). This indicates that first a chemically defined medium is superior for maximizing activation of late corn genes of *L. lactis* KW2 than complex rich media, but also that the switch from complex medium (e.g. M17G or THBG) to defined medium is critical.

[0260] Together, these results show that ComX is functional in strain KW2 when it is constitutively produced (i.e. expressed) and that growth conditions have a significant impact on the activation of late corn genes.

Example 4: Constitutive comX Expression Induces Natural Transformation

[0261] a) Acquisition of Single Mutations in the KW2 Genome from Exogenous DNA

[0262] We first tested the transfer of single point mutations in the chromosome of the ComX⁺ KW2 strain. The transforming PCR fragments used encompass the mutated *rpsL* allele of a spontaneous streptomycin-resistant (Str^r) clone of *L. lactis* subsp. *cremoris* MG1363 (*strA1* allele, also called *rpsL**). This mutated allele bears an A→T substitution at position 167 [resulting in the altered ribosomal protein S12 with mutation K56I] as compared to the sequence of the wild-type, streptomycin-sensitive MG1363. In addition to this mutation, the two *rpsL* alleles differ by a silent nucleotide substitution at position 39 (T→G). The sequence of the *rpsL* (wild-type) and *rpsL** (conferring streptomycin resistance) alleles are disclosed respectively as SEQ ID NO:23 and NO:24 (FIG. 4A). Independently of these two substi-

tutions located at positions 39 and 167, the *rpsL* alleles of KW2 and MG1363 differ by a nucleotide substitution at position 156 (A in MG1363, T in KW2). The *rpsL* allele of KW2 is disclosed as SEQ ID NO:25 (FIG. 4A). To ensure efficient recombination, the transforming PCR product also contains upstream and downstream recombination arms of ~ 1.85 kb surrounding the *strA1* mutation. Transformation assays were performed with the eight previously selected clones of the ComX⁺ reporter strain (BLD101 [pGhP32comX_{MG}]) and the control strain (BLD101 [pG⁺ host9], empty vector) using the standard protocol reported in Material and Methods. Validation of natural transformation is made by sequencing the *rpsL* region covering the point mutations from the donor DNA conferring streptomycin resistance using primers RpsL Univ UP and RpsL Univ DN. **[0263]** Remarkably, the ComX⁺ clones 02 and 04 that displayed the highest P_{comGA} activation ($\geq 7 \times 10^5$ RLU OD₆₀₀⁻¹) yielded mutation frequencies ~ 15 -fold higher than the background level of spontaneous mutation that was calculated in the absence of DNA (FIG. 4B). After subtraction of the background, a transformation rate of up to 4×10^{-5} transformants per μ g of DNA ($\sim 10^4$ transformants ml⁻¹) was obtained for clone 02 which displays the highest P_{comGA} activation. In contrast, the negative control strain had a spontaneous mutation rate of $\sim 1 \times 10^{-7}$ transformants per μ g of DNA.

[0264] The *rpsL* ORF of 10 Str^r-derivatives of cl02 was amplified by PCR and sequenced. In all cases, we observed the co-transfer of *strA1* (mutation A→T at position 167 of the *rpsL* gene) and the closely-located T→A mutation at position 156. In some cases, the T→G mutation at position 39 was also co-transferred with *strA1*. The chimeric nature of *rpsL* in some Str^r ComX⁺ derivatives of KW2 (i.e. presence of both mutations at positions 156 and 167 without the mutation at position 39) ultimately demonstrates that a recombination process occurred between the exogenous and chromosomal DNA (FIG. 4A). In contrast, this rearrangement was not observed in the *rpsL* gene of spontaneous Str^r mutants obtained in the negative control experiments (i.e. assays performed in absence of exogenous DNA, or with the control strain carrying the empty vector in presence of exogenous DNA). These results show that exogenous DNA can enter KW2 cells and be integrated in their chromosome by homologous recombination when a certain threshold of comX expression is reached.

[0265] b) Construction of Deletion Mutants by Natural Competence in *L. lactis* Subsp. *cremoris* KW2 Overexpressing comX

[0266] The previous result (Example 4, section a) strongly suggests that DNA transfer occurs in *L. lactis* KW2. The 3 mutations transferred by natural transformation are grouped on a 128-bp fragment. If a longer DNA fragment could be similarly integrated in the *L. lactis* chromosome remains to be determined.

[0267] We wondered if overlap PCR as donor DNA could equivalently allow gene insertions or gene deletions. The idea was to replace the target gene by an antibiotic resistance cassette, i.e. the chloramphenicol resistance cassette P₃₂-cat. For this purpose, a DNA fragment was constructed by overlap PCR containing the P₃₂-cat cassette flanked by two homologous arms (minimum ~ 1.5 kb) containing the upstream and downstream regions of the targeted gene.

[0268] To this end, exogenous DNA polynucleotides containing P₃₂-cat surrounded by KW2-specific recombination

arms (~1.5 kb) were assembled in vitro by overlapping PCR to target the *comEC*, *mecA*, *ciaRH*, *covRS* or *clpC* gene (see Materials and Methods for details) and transferred by natural transformation in the *ComX*⁺ strain (cl02). Validation of natural transformation is made by sequencing the targeted region (*comEC*, *mecA*, *ciaRH*, *covRS* or *clpC*, which should contain the chloramphenicol resistance cassette *P*₃₂-cat) using primers listed in Table 3.

[0269] The transformation rate observed for overlap PCR products was ~1.2×10⁻⁶ to 1.1×10⁻⁴ transformants per µg of DNA for the different overlap DNA fragments that were tested (see FIG. 5). Compared to the transformation rate observed for the exchange of a homologous DNA fragment containing only three point mutations (*rpsL** donor DNA; 8×10⁻⁴ transformants per µg of DNA), these rates are relatively high for DNA double recombination deletion/replacement.

Example 5: A KW2 Δ*comEC* Mutant is Unable of Natural Competence Transformation

[0270] To confirm that the observed horizontal DNA transfer in *ComX*⁺ KW2 cells was indeed mediated by natural competence, and not by phage transduction or conjugation, we investigated the role of the *ComEC* protein, which is essential for the uptake of transforming DNA through the cell membrane (the *comFA* gene, together with the *comGA*, *comGA*, *dprA*, *coiA*, *ssbA*, *radA*, *radC*, *recA* and *recX* genes are preceded by a *Com*-box and have been found to be activated in KW2 following constitutive *comX* expression; data not shown).

[0271] To create the Δ*comEC* strain, clone 02 of the *ComX*⁺ reporter strain, which was tested above, was grown in CDM conditions in presence of PCR products encompassing the *comEC* gene disrupted by the insertion of the chloramphenicol resistance cassette *P*₃₂-cat (see Materials and Methods). Four mutants with disrupted *comEC* (BLD102 [pGhP32*comX*_{MG}] cl01 to cl04) were validated by PCR for *P*₃₂-cat insertion in *comEC*. Transformation assays with the mutated *rpsL* allele showed that the frequencies of appearance for Str^r clones in all tested Δ*comEC* derivatives were similar to the background level of spontaneous *rpsL* mutation frequencies (<10⁻⁷) (FIG. 6). Although heterogeneity in *P*_{comGA} activation was observed between clones as previously reported for the WT *ComX*⁺ reporter strain, half of the Δ*comEC* derivative clones (i.e. cl01 and cl03) displayed maximum specific Lux activity similar to the transformable WT strains (>1.0×10⁶ RLU OD₆₀₀⁻¹) (FIG. 4B). This shows that the transformation defect in these Δ*comEC* clones does not result from a too low production of *ComX*.

[0272] Taken together, these results demonstrate that natural DNA transformation could be activated by *ComX* overexpression in *L. lactis* subsp. *lactis* KW2. Moreover, to the best of our knowledge, these data provide the first ever experimental evidence of transformation of *L. lactis* by natural competence.

Example 6: Natural Competence in Two Strains of the *L. Raffinolactis* Species

[0273] Following the positive results obtained regarding natural competence in *Lactococcus lactis* strains, other strains of the *Lactococcus* genus were tested. Two strains of *L. raffinolactis* were able to capture plasmid pGhost-Core (15 µg/300 µl) used as donor DNA: LMG13098 and

LMG14164. These results suggest that these two strains of *L. raffinolactis* are naturally competent for plasmid transformation and that, in these strains, natural competence is independent of artificial *comX*-overexpression.

[0274] The fact that another *Lactococcus* species could be transformed by competence opens additional possibilities for industrial applications.

Example 7: Transformation by Natural Competence in 2 *Lactococcus lactis* Subsp. *Lactis* Strains

[0275] Two *Lactococcus lactis* subsp. *lactis* strains, SL12651 and SL12653, carrying all the essential late *com* genes (FIG. 1) were tested. As donor DNA, PCR fragments which encompass the mutated *rpsL* allele (*rpsL**) of a spontaneous streptomycin-resistant (Str^r) clone of *L. lactis* subsp. *lactis* IL1403 was used. Cells were pre-cultured overnight in a complex medium supplemented with glucose (e.g. M17G) at 30° C. Cells were washed twice with distilled water and inoculated at an OD₆₀₀ of 0.05 in 200 µl M17G containing 25 µg mL⁻¹ donor DNA *rpsL**. After 24 hours of culture at 30° C., cells incubated or not with donor DNA were spread onto agar plates comprising the complex medium supplemented with glucose (e.g. M17G) and appropriate antibiotic (i.e. streptomycin). CFUs were counted after 48 hours of incubation at 30° C. Remarkably, SL12651 and SL12653 yielded a transformation rate of up to 1×10⁻⁶ of DNA when grown in M17G rich medium (FIG. 7A; +DNA). In contrast, the negative control in absence of donor DNA had a spontaneous mutation rate of 6×10⁻⁹ (FIG. 7A; -DNA). The transformants were validated by sequencing the *rpsL* region covering the point mutation from the donor DNA conferring streptomycin resistance.

[0276] Then, the SL12653 strain was assayed in the same conditions with variable quantity of donor DNA (0.5, 2.5, 5 and 25 µg mL⁻¹). It has been shown that the transformation rate obtained is directly correlated to the initial quantity of donor DNA, yielding up to a transformation rate of 5×10⁻⁶ (FIG. 7B).

[0277] Moreover, to confirm that the observed horizontal DNA transfer was mediated by natural competence, the *comX* gene of SL12653 was knocked-out (as described in example 5 above). Three mutants of SL12653 with disrupted *comX* gene were designed by inserting PCR products encompassing the *comX* gene disrupted by the insertion of the chloramphenicol resistance cassette *P*₃₂-cat and validated by PCR for *P*₃₂-cat insertion. Transformation assays with *rpsL** as donor DNA in all Δ*comX* clones (*ComX*⁻) showed that the frequencies of appearance of Str^r clones were similar to the background level of spontaneous mutation frequencies (FIG. 7C). These results confirm that in SL12653, the transformation is dependent on the expression of the endogenous *comX* gene.

[0278] Finally, the transformability of the SL12653 strain was also assayed by overexpressing the *comX* gene. Thus, an inducible *comX* expression plasmid [pGhPxylT*comX*_{IO}] was constructed by cloning the *comX* gene from strain *L. lactis* subsp. *lactis* IO-1 under the control of the *P*_{xyIT} promoter from strain IO-1 on the thermosensitive plasmid pG⁺host9. This plasmid is a variant of pGhPxylT*comX*_{MG} (pGIFPT001) described in David et al., 2017. The transfor-

mation procedure described in David et al (2017) was followed. In presence of xylose (1%), SL12653 [pGhPxyIT-comX_{IO}] yielded a transformation rate at least 20-fold higher than in absence of xylose, confirming that the over-expression of comX in SL12653 increased its transformability by natural competence.

Materials and Methods

[0279] Bacterial Strains, Plasmids, and Growth Conditions

[0280] The bacterial strains and plasmids used in this application are listed in Table 2.

TABLE 2

list of used bacterial strains and plasmids		
Strain or plasmid	Characteristics ^a	Source or reference
<i>E. coli</i>		
TG1	supE hsdΔ5 thi Δ(lac-proAB) F'[traD36 proAB ⁺ lacI ^q lacZΔM15]	Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
EC1000	Km ^r , recA ⁺ ; MC1000 containing a copy of the repA gene from pWV01 in its chromosome	Law, J., G. Buiet, A. Haandrikman, J. Kok, G. Venema, and K. Leenhouts. 1995. J. Bacteriol. 177: 7011-7018.
<i>L. lactis</i>		
MG1363	Laboratory strain, dairy origin	Gasson, M. J. 1983. J. Bacteriol. 154: 1-9.
KW2	Wild-type isolate from corn fermentation	Kelly, W. J., E. Altermann, S. C. Lambie, and S. C. Leahy. 2013. Front Microbiol. 4: 257.
IL1403	Laboratory strain, dairy origin	Chopin, A., M. C. Chopin, A. Moillo-Batt, and P. Langella. 1984. Plasmid 11: 260-263
IO-1	Wild-type isolate from water in the drain pit of a kitchen sink	Ishizaki A, Osajima K, Nakamura K, Katsunori K, Hara T, and Ezaki T. 1990. J. Gen. Appl. Microbiol., 36, 1-6
SL12651	Wild-type isolate from plant material (maize)	DuPont/Danisco collection
SL12653	KW2 kw2_0563::P _{comGA} [MG]-luxAB	This application
BLD101	BLD101 comEC::P ₃₂ -cat	This application
BLD102	BLD101 mecA::P ₃₂ -cat	This application
BLD107	BLD101 ciaRH::P ₃₂ -cat	This application
BLD108	BLD101 covRS::P ₃₂ -cat	This application
BLD109	BLD101 clpC::P ₃₂ -cat	This application
BLD105	BLD101 clpC::P ₃₂ -cat	This application
<i>L. raffinolactis</i>		
LMG13098	Wild-type isolate from garden carrots	LMG collection
LMG14164	Wild-type isolate from goose	LMG collection
Plasmids		
pGEM ®-T easy	Apr ^r ; cloning vector	Promega
pG ⁺ host9	Em ^r Ts	Maguin, E., H. Prevost, S. D. Ehrlich, and A. Gruss. 1996. J. Bacteriol. 178: 931-935
pGhost-Core	Em ^r Ts; pG ⁺ host9 derivative containing the Core part of the resolution site IRS recognized by the Tnpl from Tn4430	This application
pMG36eT	Em ^r ; <i>E. coli</i> - <i>L. lactis</i> shuttle vector containing the P ₃₂ constitutive promoter from <i>L. lactis</i>	Fontaine, L. and P. Hols. 2008. Appl. Environ. Microbiol. 74: 1102-1110.
pJIM4900	Em ^r Ts; pG ⁺ host9 derivative containing the luxAB genes of <i>Photobacterium luminescens</i>	E. Guédon, (laboratory collection)
pXL	Em ^r ; pTRKH2 derivative containing the luc reporter gene	Blomqvist T, Steinmoen H, Håvarstein L S. Appl Environ Microbiol. 2006. Oct; 72(10): 6751-6.
pSEUDOPusp45GFP	Em ^r ; suicide vector containing the llmg_pseudo_10(kw2_0563)::P _{usp45} gfp ⁺ insertion cassette	Overkamp, W., K. Beilharz, W. R. Detert-Oude, A. Solopova, H. Karsens, A. Kovacs, J. Kok, O. P. Kuipers, and J. W. Veening. 2013. Appl. Environ. Microbiol. 79: 6481-6490.
pUC18Cm	Ap ^r Cm ^r ; pUC18 derivative containing the P32-cat cassette	Goffin, P., F. Lorquet, M. Kleerebezem, and P. Hols. 2004. J. Bacteriol. 186: 6661-6666.
pUC18Ery	Ap ^r Em ^r ; pUC18 derivative containing an erythromycin resistance marker	van Kranenburg, R., J. D. Manugg, I. I. van Swam, N. J. Willem, and W. M. de Vos. 1997. Mol. Microbiol. 24: 387-397.
pNZ5319	Em ^r Cm ^r ; pACYC184 derivative containing the P32-cat cassette surrounded by lox sites	Lambert, J. M., R. S. Bongers, and M. Kleerebezem. 2007. Appl. Environ. Microbiol. 73: 1126-1135.
pGhPcomGAluxAB	Em ^r Ts; pG ⁺ host9 derivative containing the llmg_pseudo_10(kw2_0563)::P _{comGA} [MG]-luxAB insertion cassette	This application

TABLE 2-continued

list of used bacterial strains and plasmids		
Strain or plasmid	Characteristics ^a	Source or reference
pGhP32comX _{MG}	Em ^r Ts, pG ⁺ host9 derivative carrying comX of strain MG1363 under the control of the constitutive promoter P ₃₂	This application
pGhP32comX _{IO}	Em ^r Ts, pG ⁺ host9 derivative carrying comX of strain IO-1 under the control of the constitutive promoter P ₃₂	This application
pGhP32comX _{MG} ⁻	pGhP32comX _{MG} derivative carrying a P _{comGA[MG]} -luc fusion	This application
pGhP32comX _{IO} ⁻	pGhP32comX _{IO} derivative carrying a P _{comGA[IO]} -luc fusion	This application
pGEMrpsL*	Ap ^r , pGEM ®-T easy derivative carrying the rpsL* gene (strA1 allele)	This application
pUCcomECcat	Ap ^r Em ^r Cm ^r , pUC18Ery derivative allowing the insertion of P ₃₂ -cat at the comEC locus	This application
pGhPxylTcomX _{IO}	Em ^r Ts, pG ⁺ host9 derivative carrying comX of strain IO-1 under the control of the inducible promoter P _{xyIT} from IO-1	This application

^a Em^r, Ap^r, Cm^r and Ts: erythromycin, ampicillin, chloramphenicol resistance and thermo-sensitive RepA protein, respectively.

[0281] *Escherichia coli* was grown with shaking at 37° C. in Lysogeny-Broth (LB) broth. Plasmids derived from pMG36e and pG⁺host9 were constructed in *E. coli* strains TG1 and EC1000, respectively. *L. lactis* and *L. raffinolactis* were cultivated in M17 (Becton, Dickinson, and Company), Todd Hewitt broth (THB) (Becton, Dickinson, and Company) or CDM at 30° C. without agitation. M17 and THB were supplemented with 0.5% (w/v) of glucose (M17G and THBG, respectively). Solid agar plates were prepared by adding 2% (w/v) agar to the medium. When required, 5 µg ml⁻¹ of erythromycin, 1 mg ml⁻¹ of streptomycin, and/or 10 µg ml⁻¹ of chloramphenicol were added to the medium for *L. lactis* and *L. raffinolactis*; and 250 µg ml⁻¹ of erythromycin, 250 µg ml⁻¹ of ampicillin, 10 µg ml⁻¹ of chloramphenicol for *E. coli*.

[0282] Detection of Absorbance and Luminescence.

[0283] Growth (OD₆₀₀) and luciferase (Lux) activity were monitored at 10-minutes intervals in a Varioskan Flash multi-mode reader (ThermoFisher). The luciferase activity is expressed in relative light units (RLU) and the specific luciferase activity in RLU OD₆₀₀⁻¹.

[0284] DNA Techniques and Electrotransformation

[0285] General molecular biology techniques were performed according to the instructions given by Sambrook et al. (1989). Electrotransformation of *E. coli* and *L. lactis* was performed as previously described. The electrotransformed cells of *L. lactis* were immediately resuspended in 1 ml of M17G and incubated for 6 hours at 30° C. Chromosomal DNAs of *L. lactis* were prepared as previously described. PCRs were performed with Phusion DNA polymerase (NEB) in a GeneAmp PCR system 2400 (Applied Biosystems). The primers used in this application are listed in Table 3.

TABLE 3

list of primers	
Primer name	Sequence (5'-3')
Primers used for the construction of the constitutive comX expression plasmid pGhP32comX _{MG/IO} :	
BID_ComXSDDLlCup	AAAAGAGCTCAATTATGAAAAAGAGG
BID_ComXSDDLlCdown	AAAAGAGCTCAATTATGAAAAAGAGG
BID_ComXSDDLlLup	AAAAGAGCTCATAAAAGGAGAACTTTCC
BID_ComXSDDLlLdown	AAAAGAGCTCATAAAAGGAGAACTTTCC
BID_pMGP32UpMfeI	ATATCAATTGGTCCTCGGGATATGATAAG
BID_pMGTerDown	GACTTTGAACCTCAACTCC

TABLE 3-continued

list of primers	
Primer name	Sequence (5'-3')
Primers used for the construction of the P _{comGA[MG]} -luxAB reporter strain BLD101:	
BID_LuxLLCf1	ATAGTCTCGAGTTTAAGCAATTGAATCGCTAG
BID_LuxLLCr1	GCAAAAAGTTTCCAAATTCATACTAGAAATATACGCAATTTG
BID_LuxLLCf2	CAAATTGCGTATATTCTAGTATGAAATTTGGAACTTTTTCG
BID_LuxLLCr2	GCGAAAGGATCCCTATTAGGTATATTCCATGTGG
BID_P3pseudoLLC	GCTCCCTCGAGGGCGGCTCTGTTGGATTAATATATGG
Primers used for the construction of portable luc reporter vectors:	
BID_LucLLCr1	CTTTATGTTTTTGGCGGATCTCATACTAGAAATATACGCAATTTG
BID_LucLLCf2	CAAATTGCGTATATTCTAGTATGAGATCCGCCAAAACATAAAG
BID_LucLLCr2	GCGAAAGGATCCTTACAATTTGGGCTTTCCG
BID_PcomGALLCF1*	AAAACCCGGGTTTAAGCAATTGAATCGCTAG
BID_PcomGALLLF1*	5' AAAACCCGGGAAATAAATGGCTACAAAATT
BID_lucR1*	AAAACGGCCGTTACAATTTGGGCTTTCCG
BID_luxLLLf1	ATAGTCTCGAGAAATAAATGGCTACAAAATT
BID_lucLLLf1	CTTTATGTTTTTGGCGGATCTCATACTAGAAATATACGCAATAATC
BID_lucLLLf2	GATTATTTGCGTATAGTCTAGTATGAGATCCGCCAAAACATAAAG
BID_lucLLLf2	GCGAAAGGATCCTTACAATTTGGGCTTTCCG
Primers used for the construction of pGhost-Core	
DD-pGhost-CoreUp	AGCTTCCTAATACACACAATTAATATTGTGTGTATTATTG
DD-pGhost-CoreDW	AATTCAATAATACACACAATATTAATTGTGTGTATTAGGA
Primers used for rpsL sequencing:	
RpsL Univ UP	ATGCCTACAATTAACCAAT
RpsL Univ DN	CACCGTATTTAGAACGG
LR_RpsL Univ UP	ATGCCTACTATTAACCAAT
LR_RpsL Univ DN	TACCGTATTTAGAACGG
Primers used for rpsL amplification:	
BID_LLCdACrpsL	AGTAGTATCAGCACTGACAGC
BID_LLIcfusArpsL	ACACCTTTGTTCTTGAAGG
primers used for the construction of the comEC disruption mutant:	
BID_ComeCLLCUp	AAAGAGCTCAAAATAAAAATGAAATTATGG
BID_ComeCLLCDown	AAAGCTAGCGGAAAAAATTGTGAATTAC
BID_CatUpSpeI	AAAACTAGTGAGTTTAAATTCGGTCTCTCG
BID_CatDownSpeI	AAAACTAGGTACAGTCGGCATTATCTCAT
Primers used for the construction and validation of the mecA deletion mutant:	
BID_fgt01FmecArec	CTTTAATGATGAATGATTG
BID_fgt01RVmecArec	CTATTAATCTTATCATATCCCGAGGATCCATATAACTATATGAAACC
BID_fgt02Fcat	TCCTCGGATATGATAAGATTAATAG

TABLE 3-continued

list of primers	
Primer name	Sequence (5'-3')
BID_fgt02RVcat	TCTCATATTATAAAAGCCAGTCATTAG
BID_fgt03FmecArec	CTAATGACTGGCTTTTATAATATGAGACTTAGAAAAATCTAAATATGGTTG
BID_fgt03RVmecArec	GAAGATTTTAAATTTCAAGTGTAG
BID_mecAKOF	TCAGTACCGAAAAACGAATG
BID_mecAKORV	ATTTACCAGTTCGGTTAGG
Primers used for the construction and validation of the ciaRH deletion mutant:	
BID_ciaRHUPF	TAACAATGATACAGAAGATG
BID_ciaRHUPRVRec	CTATTAATCTTATCATATCCCGAGGATATTTTGTCTTGACTAGG
BID_fgt02Fcat	TCCTCGGGATATGATAAGATTAATAG
BID_fgt02RVcat	TCTCATATTATAAAAGCCAGTCATTAG
BID_ciaRHDownFRec	CTAATGACTGGCTTTTATAATATGAGAGAGAGAAAAAATTACTGAC
BID_ciaRHDownRV	AAAATCTGTGTAAGACTGTG
BID_ciaRHKODiagF	AAGATAAGGCAGTTGAAATG
BID_ciaRHKODiagRy	TCACCATGTGAATAAAGTCC
Primers used for the construction and validation of the covRSdeletion mutant:	
BID_covRSfgt01F	CAAAAATGTGAAGCTTATC
BID_covRSfgt01RVRec	CTATTAATCTTATCATATCCCGAGGATGCATAATTCGATTTT
BID_fgt02Fcat	TCCTCGGGATATGATAAGATTAATAG
BID_fgt02RVcat	TCTCATATTATAAAAGCCAGTCATTAG
BID_covRSfgt03FRec	TAATGACTGGCTTTTATAATATGAGACTATTTATCTGCTCATTTT
BID_covRSfgt03RV	GAGCTTTTTCAAATCTTC
BID_covRSKOFdiag	GAAGTGATGAATGAGATG
BID_covRSKORVdiag	CTTTCTCATCAATTGAGAC
Primers used for the construction and validation of the clpC deletion mutant:	
BID_clpCUPF	CTTTGGGTCTAATTTATC
BID_clpCUPRVRec	CTATTAATCTTATCATATCCCGAGGACGTTGGTGATATTTTAC
BID_fgt02Fcat	TCCTCGGGATATGATAAGATTAATAG
BID_fgt02RVcat	TCTCATATTATAAAAGCCAGTCATTAG
BID_clpCDownFRec	CTAATGACTGGCTTTTATAATATGAGATAGAAATAAGGAAAGGAC
BID_clpCDownRV	TTGCTTTAAGGATAGTTTC
BID_clpCFdiag	AGAAGCCAATAATGACGATG
BID_clpCRVdiag	AGAATTCTGATGATGCACAGTC
Primers used for the construction of the inducible comX expression plasmid pGhPxylTcomX _{IO} :	
FT_pGhPxylcomXIOsacllrv	AGCGCCGCGGTGGGATCCTCTAGAGTC
FT_pGhPxylcomX	CTGCAGGCATGCACATCATCAACTTGAAGGG
FT_PxylTIOsacllfw	CCCACCGCGGTGGAGATACGAACAAATTAG

TABLE 3-continued

list of primers	
Primer name	Sequence (5'-3')
FT_PxylTIorv	GATAGTAACCTCTTAATTTTATTTCG
FT_comXIorecfw	GCAAATAAAATTAAGGAGTTACTATCATGACATATTACTTGAAGAAGAGGAT TTTG
FT_comXIorecrv	CCTTCAAGTTGATGATGTGCATGCCTGCAGTCACTCTTCGTCTTC
Primers used for the construction and validation of the SL12653-comX deletion mutant	
FT_comXlocusfw	TGACCATTGTACACAAGCCTATATCCT
FT_comXreclv	CGCCCTTATGGGATTATCTTCTTACTTCGTTCTTTGCATAACTTCGTCTTA AT
Uplox66	TAAGGAAGATAAATCCATAAGG
Dnlox71	TTACGTTACTAAAGGGAATGTA
FT_comXreclv	TCTACATTCCTTTAGTAACGTGAACCATGACCATTTATAGGTTAGATGTTT ATG
AR_comxDNspecR	CGGTGTTCTCCATATATCTACGC
FT_PxylcomXfw	CGCTAAACTCAACAGGTGATCCGATTG

[0286] Construction of Plasmid pGhP32comX_{MG}

[0287] As a representative of the *cremoris* subspecies, the comX gene from the laboratory strain MG1363 was initially chosen. ComX proteins of this subspecies are highly conserved with at least 98% of identity. The comX gene was amplified by PCR using primers BID_ComXSDDLlCup/BID_ComXSDDLlCdown and inserted into plasmid pMG36eT under the control of the constitutive P₃₂ promoter by SacI/PstI cloning, yielding plasmid pMGP32comX_{MG}. The P₃₂-comX_{MG} fusion from pMGP32comX_{MG} was amplified by PCR with primers BID_pMGP32UpMfeI/BID_pMGTerDown, digested by MfeI/KpnI, and cloned in the EcoRI/KpnI-digested thermosensitive pG⁺host9 vector. The resulting plasmid was named pGhP32comX_{MG}.

[0288] Construction of Plasmid pGhP32comX_{IO}

[0289] As a representative of the *lactis* subspecies, the comX gene from the IO-1 strain was chosen. The comX gene was amplified by PCR using primers BID_ComXSDDLlCup/BID_ComXSDDLlCdown and inserted into plasmid pMG36eT under the control of the constitutive P₃₂ promoter by SacI/PstI cloning, yielding plasmid pMGP32comX_{IO}. The P₃₂-comX_{IO} fusion from pMGP32comX_{IO} was amplified by PCR with primers BID_pMGP32UpMfeI/BID_pMGTerDown, digested by MfeI/KpnI, and cloned in the EcoRI/KpnI-digested thermosensitive pG⁺host9 vector. The resulting plasmid was named pGhP32comX_{IO}.

[0290] Construction of Plasmid pGhost-Core

[0291] The Core part of the resolution site (IRS) recognized by the TnpI recombinase from Tn4430 was assembled by using the complementary primers DD-pGhost-CoreUp/DD-pGhost-CoreDW. The resulting DNA fragment was cloned between HindIII and EcoRI sites in plasmid pG⁺host9. The resulting plasmid, named pGhost-Core, was transformed in *E. coli* harbouring plasmid pGIV004 (TnpI⁺)

for obtaining multimeric forms (Vanhooft V, Galloy C, Agaisse H, Lereclus D, Revet B, Hallet B. Mol Microbiol. 2006 May; 60(3):617-29).

[0292] Construction of P_{comGA[MG]}-luxAB Reporter Strain BLD101

[0293] The P_{comGA[MG]} promoter was amplified by PCR from chromosomal DNA of *L. lactis* MG1363 (identical nucleotide sequence between MG1363 and KW2) with primers BID_LuxLLCf1/BID_LuxLLCr1 (PCR1 product). The luxAB genes were amplified by PCR from plasmid pJIM4900 with primers BID_LuxLLCf2/BID_LuxLLCr2 (PCR2 product). The P_{comGA[MG]}-luxAB fusion was created by overlapping PCR using PCR1 and PCR2 products and primers BID_LuxLLCf1/BID_LuxLLCr2. The resulting fusion was cloned in plasmid pSEUDOPusp45GFP using restriction enzymes XhoI and BamHI, yielding plasmid pSEUDOPusp45PcomGAluxAB. In order to remove the P_{usp45} promoter, the entire vector except the P_{usp45} promoter was amplified by inverse PCR with primers BID_P3pseudoLLC/BID_LuxLLCf1 and self-ligated after XhoI digestion, leading to plasmid pSEUDOPcomGAluxAB. The insertion cassette limg_pseudo_10::P_{comGA[MG]}-luxAB was excised from plasmid pSEUDOPcomGAluxAB and cloned into the pG⁺host9 thermosensitive vector using restriction enzymes KpnI/EagI. The resulting plasmid pGhPcomGAluxAB was then electro-transformed in strain KW2 and used to integrate the P_{comGA[MG]}-luxAB cassette at locus kw2_0563 (limg_pseudo_10 in MG1363) by double homologous recombination, resulting in the reporter strain KW2 kw2_0563::P_{comGA[MG]}-luxAB (strain BLD101).

[0294] Construction of Portable Luc Reporter Systems

[0295] The P_{comGA[MG]} promoter was amplified by PCR from chromosomal DNA of *L. lactis* MG1363 with primers BID_LuxLLCf1/BID_LucLLCr1 (PCR1 product). The luc gene was amplified by PCR from plasmid pXL with primers BID_LucLLCf2/BID_LucLLCr2 (PCR2 product). The P_{comGA[MG]}-luc fusion was created by overlapping PCR

using PCR1 and PCR2 products and primers BID_LuxLLCf1/BID_LucLLCr2. The resulting fusion was cloned in plasmid pSEUDOPusp45GFP using restriction enzymes XhoI and BamHI, yielding plasmid pSEUDOPusp45PcomGAluc. In order to remove the P_{usp45} promoter, the entire vector except the P_{usp45} promoter was amplified by inverse PCR with primers BID_P3pseudoLLC/BID_LuxLLCf1 and self-ligated after XhoI digestion, leading to plasmid pSEUDOPcomGAluc. The reporter cassette $P_{comGA[MG]}$ -luc was amplified by PCR from pSEUDOPcomGAluc (primers BID_PcomGALLCF1*/BID_lucR1*) and cloned between XmaI and EagI into the pGhP32comX_{MG} plasmid. The resulting reporter plasmid was named pGhP32comX_{MG}- $P_{comGA[MG]}$ -luc.

[0296] The $P_{comGA[IO]}$ promoter was amplified from the IO-1 chromosome (primers BID_luxLLLf1/BID_lucLLLr1) and the luciferase gene (luc) was amplified from plasmid pXL (primers BID_lucLLLf2/BID_lucLLLr2). The cassette $P_{comGA[IO]}$ -luc was created by overlapping PCR with primers BID_luxLLLf1/BID_lucLLLr2. The cassette $P_{comGA[IO]}$ -luc was then amplified from the overlapping PCR product with primers BID_PcomGALLLF1*/BID_lucR1* for XmaI/EagI cloning into pGhP32comX_{IO}. The resulting reporter plasmid was named pGhP32comX_{IO}- $P_{comGA[IO]}$ -luc.

[0297] Isolation of a rpsL Mutant Conferring Resistance to Streptomycin

[0298] Spontaneous streptomycin-resistant MG1363 clones were isolated on 1 mg ml⁻¹ streptomycin-containing plates. After the sequencing of the rpsL gene with primers RpsL Univ UP/RpsL Univ DN, one spontaneous mutant resulting in a mutation (K56I) into the ribosomal protein S12 that was previously shown to confer resistance to streptomycin was selected (FIG. 6). A 3.7-kb fragment containing the rpsL mutated gene (strA1 allele) was amplified by PCR with primers BID_LLcdacARpsL/BID_LLlcfusARpsL and cloned into the pGEM®-T easy vector (Promega), yielding plasmid pGEMrpsL*. This plasmid was used as template to generate the 3.7-kb PCR product with primers BID_LLcdacARpsL/BID_LLlcfusARpsL that was used as donor DNA in natural transformation assays of strain KW2.

[0299] Standard Natural Transformation Assay

[0300] The BLD101 reporter strain carrying the pGhP32comX_{MG} plasmid (BLD101 [pGhP32comX_{MG}]) was grown overnight in M17G at 30° C. Then, 1.5 ml of the pre-culture was diluted in 8.5 ml of fresh M17G medium to restart the culture. After 2 hours of growth, cells were washed twice in distilled water and OD₆₀₀ was adjusted to 0.05 in CDM containing erythromycin (5 µg ml⁻¹) and supplemented with either 5% (v/v) glycerol or 5% (w/v) mannitol used as potential osmo-stabilizers. Typically, 5 µg of DNA was added in 300 µl of inoculated medium and the culture was further incubated during 6 hours at 30° C. Cells were then spread on M17G agar plates supplemented with appropriate antibiotics and CFUs were counted after 48 hours of incubation. The transformation frequency was calculated as the number of antibiotic-resistant CFU ml⁻¹ divided by the total number of viable CFU ml⁻¹. In the case of streptomycin-resistant transformants, antibiotic-resistant CFU ml⁻¹ corresponds to the number of transformants obtained in presence of DNA less the number of spontaneous transformants obtained in conditions where no DNA is added in the culture. The transfer of the mutation conferring streptomycin resistance was confirmed by DNA sequencing

of the rpsL gene after its amplification by PCR using primers RpsL Univ UP/RpsL Univ DN.

[0301] Disruption of comEC by Natural Transformation

[0302] A comEC-containing DNA fragment of ~3.2 kb was amplified by PCR with primers BID_ComeCLLCUp/BID_ComeCLLCDown. Then, the PCR product was digested by SacI/NheI and cloned into the SacI/XbaI-digested suicide plasmid pUC18Ery (van Kranenburg et al., 1997), yielding plasmid pUCcomEC. To generate a comEC disruption cassette that allows the selection of double crossing-over recombinants, the P₃₂-cat fusion conferring resistance to chloramphenicol was cloned in the middle of the comEC gene. For this purpose, the P₃₂-cat cassette was amplified by PCR from plasmid pNZ5319 (Lambert et al., 2007, Appl. Environ. Microbiol. 73:1126-1135) with primers BID_CatUpSpeI/BID_CatDownSpeI. The amplification product was digested by SpeI and cloned into the XbaI-digested pUCcomEC, yielding plasmid pUCcomECcat. This suicide plasmid was used to generate high quantity of donor DNA by PCR amplification for comEC disruption by natural transformation. The insertion of the P₃₂-cat cassette in the comEC gene of KW2 transformants was validated by PCR (primers in Table 3).

[0303] Deletion of mecA, ciaRH, covRS, and clpC Genes by Natural Transformation

[0304] The mecA, ciaRH, covRS, and clpC genes were similarly inactivated by the exchange of their ORFs by the P₃₂-cat cassette using double crossing-over events. For this purpose, overlapping PCR products containing the P₃₂-cat cassette flanked by two recombination arms of ~1.5 kb (upstream and downstream homologous regions) were generated as previously reported. Briefly, upstream, downstream, and P₃₂-cat fragments were separately amplified by PCR, purified, mixed in equimolar concentration, and assembled by overlapping PCR by using the most external primers (see list of primers in Table 3). 5 µg of the obtained overlapping PCR product was used as donor DNA for natural transformation of strain BLD101 [pGhP32comX_{MG}]. The correct insertion of the P₃₂-cat cassette in each targeted locus of the KW2 transformants was validated by PCR (see list of primers in Table 3). To obtain the final mutant strains, the thermosensitive vector pGhP32comX_{MG} was cured by growing the strains overnight at 37° C. without erythromycin. The cultures were subsequently diluted and plated on M17G agar without erythromycin at 30° C. The resulting colonies were streaked in parallel on M17G plates with and without erythromycin. Absence of plasmid pGhP32comX_{MG} in Ery^S clones was validated by PCR.

[0305] Induction of Natural Competence in *Lactococcus raffinolactis*

[0306] Wild-type *Lactococcus raffinolactis* (i.e., *L. raffinolactis* strains which have not been previously engineered for the overproduction of the comX gene) were grown overnight in M17G at 30° C. 1.5 ml of the pre-culture was diluted in 8.5 ml of fresh M17G medium to restart the culture. After 2 hours of growth, cells were washed twice in distilled water and OD₆₀₀ was adjusted to 0.05 in CDM supplemented with either 5% (v/v) glycerol or 5% (w/v) mannitol used as potential osmo-stabilizers. 15 µg of plasmid pGhost-Core was added in 300 µl of inoculated medium and the culture was further incubated during 6 hours at 30° C. Cells were then spread on M17G agar plates supplemented with appropriate antibiotics and CFUs were counted

after 48 hours of incubation. The transformation frequency was calculated as the number of antibiotic-resistant CFU ml⁻¹ divided by the total number of viable CFU ml⁻¹.

[0307] Natural Competence in *Lactococcus lactis* Subsp *Lactis* SL12651 and SL12653 Strains

[0308] The *L. lactis* subsp. *lactis* SL12653 and 12651 strains were grown overnight at 30° C. Cells were washed twice in distilled water and OD₆₀₀ was adjusted to 0.05 in M17G. Typically, 5 µg of donor DNA was added in 200 µl of inoculated medium (25 µg/ml) and the culture was further incubated during 24 hours at 30° C. Cells were then spread on M17G agar plates supplemented with appropriate antibiotics and CFUs were counted after 48 hours of incubation at 30° C. The transformation frequency calculated exactly as described above (see Standard natural transformation assay).

[0309] The same experiments were done in SL12653 with various concentrations of donor DNA (0.5, 2.5, 5 and 25 µg/ml)

[0310] Construction of Plasmid pGhPxylTcomXIO

[0311] As a representative of the *lactis* subspecies, the comX gene and the promoter of the xylT gene from the IO-1 strain were chosen. The comX gene was amplified by PCR using primers FT_comXIOrecf and FT_comXIOrecr (PCR1), both containing overlapping sequences. The xylT promoter region was amplified by PCR using primers FT_PxylTIOsacllfw and FT_PxylTIOrv (PCR2). The carrying vector was amplified from plasmid pGhP32comX_{MG} and amplified by PCR using primers FT_pGhPxylcomXIOsacllr and FT_pGhPxylcomX (PCR3). The three PCR products were purified, mixed in an equimolar concentration and assembled by overlapping PCR using the most external primers, containing a SacII restriction site. The amplification product was digested by SacI and self-ligated. The resulting plasmid was named pGhPxylTcomX_{IO}.

[0312] Transformation Assay in SL12653 Mutants Deleted for the comX Gene

[0313] The comX gene of SL12653 was inactivated by exchange of their ORF by the P₃₂-cat cassette using double crossing-over events. For this purpose, overlapping PCR products containing the P₃₂-cat cassette flanked by two recombination arms of ~1.5 kb (upstream and downstream homologous regions) were generated as previously reported. Briefly, upstream, downstream, and the P₃₂-cat fragments were separately amplified by PCR, purified and mixed in equimolar concentration, and assembled by overlapping PCR by using the most external primers (see primers in Table 3). 5 µg of the obtained PCR product was used as donor DNA for natural transformation of strain SL12653 [pGhPxylTcomX_{IO}] (ComX⁺). The correct insertion of the P₃₂-cat cassette in the targeted locus of SL12653 transformants was validated by PCR (see primers in Table 3). To obtain the final mutant strains, the thermosensitive vector pGhPxylTcomX_{IO} was cured by growing the strains overnight at 37° C. without erythromycin.

[0314] The cultures were subsequently diluted and plated on M17G agar without erythromycin at 30° C. The resulting colonies were streaked in parallel on M17G plates with and without erythromycin. Absence of plasmid pGhPxylTcomX_{IO} in Ery^S clones was validated by PCR. Thus, 3 ΔcomX clones of SL12653 were obtained.

[0315] Xylose-Induced Natural Transformation in SL12653.

[0316] The *L. lactis* subsp. *lactis* SL12653 [pGhPxylTcomX_{IO}] was grown overnight at 30° C. Cells were washed

twice in distilled water and OD₆₀₀ was adjusted to 0.05 in M17 supplemented with 1% (w/v) xylose. Typically, 5 µg of DNA was added in 200 µl of inoculated medium and the culture was further incubated during 24 hours at 30° C. Cells were then spread on M17G agar plates supplemented with appropriate antibiotics and CFUs were counted after 48 hours of incubation at 30° C. The transformation frequency was calculated exactly as described above (see Standard natural transformation assay).

[0317] All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology, biochemistry, microbiology, bacteriology, or related fields are intended to be within the scope of the following claims.

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Leu Leu Glu Glu Asn His Pro Ser Thr Asn Ile Tyr Thr Lys Phe Lys
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Val Lys Tyr His Gln His Leu Ile Asp Glu Leu Arg His Ser Tyr Ala
65          70          75          80
Lys Lys Arg Leu His Asp His Phe Val Gly Leu Asp Ile Tyr Glu Cys
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 <211> LENGTH: 159
 <212> TYPE: PRT
 <213> ORGANISM: Lactococcus piscium

<400> SEQUENCE: 12

Met	Glu	Thr	Leu	Glu	Ala	Met	Leu	Lys	Asn	Ile	Glu	Pro	Ile	Ile	Met
1				5					10					15	

-continued

Asn	Cys	Gln	Lys	Met	Ala	Lys	Ile	Pro	Ser	Trp	Asp	Ile	Asp	Asp	Tyr
			20					25					30		
Met	Gln	Glu	Gly	Arg	Ile	Ile	Ala	Leu	Asp	Leu	Tyr	Asn	Gln	Leu	Ala
		35					40					45			
Glu	Arg	Met	Glu	Thr	Asp	Glu	Val	Asn	Phe	Tyr	Val	Tyr	Phe	Lys	Val
		50				55					60				
Arg	Tyr	Thr	Cys	Phe	Leu	Ile	Asp	Thr	Tyr	Arg	Lys	Thr	Asn	Ala	Phe
65					70					75					80
Lys	Arg	Lys	Phe	Asp	Gln	Pro	Ile	Tyr	Leu	Asp	Val	Ser	Glu	Ala	Phe
			85					90						95	
Asn	Leu	Tyr	Asp	His	Lys	Gln	Asn	Val	Ala	Asp	Asn	Val	Met	Tyr	Thr
			100					105					110		
Leu	Leu	His	Gln	Glu	Ile	Leu	Asp	Ile	Leu	Thr	Pro	Val	Glu	Ile	Gln
		115					120					125			
Thr	Leu	Asn	Ala	Leu	Lys	Arg	Gly	Glu	Lys	Val	Asp	Arg	Asn	Lys	Lys
	130					135					140				
Phe	Arg	Ile	Lys	Lys	Lys	Ile	Ile	Asn	Tyr	Ile	Asn	Gln	Ile	Phe	
145					150					155					

<210> SEQ ID NO 13
 <211> LENGTH: 486
 <212> TYPE: DNA
 <213> ORGANISM: Lactococcus garvieae

<400> SEQUENCE: 13

atggagcata atttagatat ggagcagctg gaagaaattt ttcattctgt ccaacatatt	60
gtgtggaaga acagtcgttt gattccgata aatttttgga cgtttgatga ctatcagcag	120
gaagggcgct tggattata cgatttgctg ggagatggtg tgacgcaaag gaacttattt	180
tgccatttta aggtacgcta taagcagaga cttattgata ttaaaagaag ggagcgggct	240
tttaaaagggt gttttgattg cgggactggc ttagatatat acgaatatc tgatgctcta	300
aaggggaaag cagccagtcc agaacatata ctgatttctg gaagtttact tgaagaagtt	360
tttgaaaact taaatttacg ctaccgacgg ctctcaaaa gttacctgc cgcgatgaa	420
ttgcaccgta tggaaaagta tcgtttgaag gaaaaataa cgaatatatt atatgaacag	480
cagtga	486

<210> SEQ ID NO 14
 <211> LENGTH: 161
 <212> TYPE: PRT
 <213> ORGANISM: Lactococcus garvieae

<400> SEQUENCE: 14

Met	Glu	His	Asn	Leu	Asp	Met	Glu	Gln	Leu	Glu	Glu	Ile	Phe	His	Ser
1				5						10				15	
Val	Gln	His	Ile	Val	Trp	Lys	Asn	Ser	Arg	Leu	Ile	Pro	Ile	Asn	Phe
		20						25					30		
Trp	Thr	Phe	Asp	Asp	Tyr	Gln	Gln	Glu	Gly	Arg	Leu	Val	Leu	Tyr	Asp
		35						40				45			
Leu	Leu	Gly	Asp	Gly	Val	Thr	Gln	Arg	Asn	Leu	Phe	Cys	His	Phe	Lys
	50					55					60				
Val	Arg	Tyr	Lys	Gln	Arg	Leu	Ile	Asp	Ile	Lys	Arg	Arg	Glu	Arg	Ala
65					70					75					80

-continued

Phe	Lys	Arg	Gly	Phe	Asp	Cys	Gly	Thr	Gly	Leu	Asp	Ile	Tyr	Glu	Tyr
			85						90					95	
Ser	Asp	Ala	Leu	Lys	Gly	Lys	Ala	Ala	Ser	Pro	Glu	His	Ile	Leu	Ile
			100					105					110		
Ser	Gly	Ser	Leu	Leu	Glu	Glu	Val	Phe	Glu	Asn	Leu	Asn	Leu	Arg	Tyr
			115				120					125			
Arg	Arg	Leu	Leu	Lys	Ser	Tyr	Leu	Ala	Gly	Asp	Glu	Leu	His	Arg	Met
			130			135					140				
Glu	Lys	Tyr	Arg	Leu	Lys	Glu	Lys	Ile	Thr	Asn	Ile	Leu	Tyr	Glu	Gln
			145		150					155					160

Gln

<210> SEQ ID NO 15
 <211> LENGTH: 489
 <212> TYPE: DNA
 <213> ORGANISM: Lactococcus garvieae

<400> SEQUENCE: 15

atggcagaaa ataatttaga taaagaacag cttgaagagt tattccattc acttcaacat	60
attgtttgga agaacagtca tttaattaaa ataaattttt ggacaatgga tgattatcag	120
caagaagggc gactggtttt ataccagtta cttgaagatg gcgtgacaca ggaaaaacta	180
ttttgccatt ttaaagtgcg atataagcaa cggttgattg atataaaaag acgagaaaga	240
gcatttaagc ggggttttga ttgtggggct ggtttagata tatatgagta ttctgatgcc	300
ctgaaaggca aagctaccag tcctgaatat aacttaattt cagttacttt acttgaagag	360
gttcacataa gtttgagttt gagataccgc aattttattgg agaatcatct gtcaggagtg	420
gagttgcatc gaatggaaaa ataccgttta aaggaaaaaa tcaagagaat actctatgaa	480
gaagaatga	489

<210> SEQ ID NO 16
 <211> LENGTH: 162
 <212> TYPE: PRT
 <213> ORGANISM: Lactococcus garvieae

<400> SEQUENCE: 16

Met	Ala	Glu	Asn	Asn	Leu	Asp	Lys	Glu	Gln	Leu	Glu	Glu	Leu	Phe	His
1			5						10					15	
Ser	Leu	Gln	His	Ile	Val	Trp	Lys	Asn	Ser	His	Leu	Ile	Lys	Ile	Asn
			20					25					30		
Phe	Trp	Thr	Met	Asp	Asp	Tyr	Gln	Gln	Glu	Gly	Arg	Leu	Val	Leu	Tyr
			35				40					45			
Gln	Leu	Leu	Glu	Asp	Gly	Val	Thr	Gln	Glu	Lys	Leu	Phe	Cys	His	Phe
			50			55					60				
Lys	Val	Arg	Tyr	Lys	Gln	Arg	Leu	Ile	Asp	Ile	Lys	Arg	Arg	Glu	Arg
			65		70				75					80	
Ala	Phe	Lys	Arg	Gly	Phe	Asp	Cys	Gly	Ala	Gly	Leu	Asp	Ile	Tyr	Glu
				85					90					95	
Tyr	Ser	Asp	Ala	Leu	Lys	Gly	Lys	Ala	Thr	Ser	Pro	Glu	Tyr	Asn	Leu
			100				105						110		
Ile	Ser	Val	Thr	Leu	Leu	Glu	Glu	Val	His	Gln	Ser	Leu	Ser	Leu	Arg
			115				120					125			
Tyr	Arg	Asn	Leu	Leu	Glu	Asn	His	Leu	Ser	Gly	Val	Glu	Leu	His	Arg

-continued

130	135	140	
Met Glu Lys Tyr Arg Leu Lys Glu Lys Ile Lys Arg Ile Leu Tyr Glu			
145	150	155	160

Glu Glu

<210> SEQ ID NO 17
 <211> LENGTH: 486
 <212> TYPE: DNA
 <213> ORGANISM: Lactococcus garvieae

<400> SEQUENCE: 17

atggagcata atttagatat ggagcagctg gaagagatat ttcattctgt tcaacatatt	60
gtatggaaga atagtcggtt gattccgata aatttttggg cgatagatga ctatcagcag	120
gaaggcggtt tgggtattata tgatttactt gaggatgggtg tgacacaaag aaaacttttt	180
tgccatttta aagtacgtta taagcagaga cttattgata ttaaaagaag ggagcgggct	240
tttaaaaggg gttttgactg tgggactggg ctagatattt acgaatatc agatgcttta	300
aaagaaaag tagccagtc agaacatact ctgatttctg gcagtttgct tgaagaagtt	360
ttagaaaact taaatttacg ctaccgtgct cttcttaaaa gttaccttgc tggatgatgaa	420
ctgcatcgaa tggaaaaaca tcgtttgaaa gaaaaataa taaaaatatt atatgatgaa	480
cagtga	486

<210> SEQ ID NO 18
 <211> LENGTH: 161
 <212> TYPE: PRT
 <213> ORGANISM: Lactococcus garvieae

<400> SEQUENCE: 18

Met Glu His Asn Leu Asp Met Glu Gln Leu Glu Glu Ile Phe His Ser	
1	15
Val Gln His Ile Val Trp Lys Asn Ser Arg Leu Ile Pro Ile Asn Phe	
20	30
Trp Thr Ile Asp Asp Tyr Gln Gln Glu Gly Arg Leu Val Leu Tyr Asp	
35	45
Leu Leu Glu Asp Gly Val Thr Gln Arg Lys Leu Phe Cys His Phe Lys	
50	60
Val Arg Tyr Lys Gln Arg Leu Ile Asp Ile Lys Arg Arg Glu Arg Ala	
65	80
Phe Lys Arg Gly Phe Asp Cys Gly Thr Gly Leu Asp Ile Tyr Glu Tyr	
85	95
Ser Asp Ala Leu Lys Gly Lys Val Ala Ser Pro Glu His Thr Leu Ile	
100	110
Ser Gly Ser Leu Leu Glu Glu Val Leu Glu Asn Leu Asn Leu Arg Tyr	
115	125
Arg Ala Leu Leu Lys Ser Tyr Leu Ala Gly Asp Glu Leu His Arg Met	
130	140
Glu Lys His Arg Leu Lys Glu Lys Ile Ile Lys Ile Leu Tyr Asp Glu	
145	160

Gln

<210> SEQ ID NO 19
 <211> LENGTH: 438

-continued

<212> TYPE: DNA

<213> ORGANISM: *Lactococcus fujiensis*

<400> SEQUENCE: 19

```

tgaaaccga tcgtttcaaa atctatgaga acattaaaaa tcaatttttg gactacagag    60
gattatcatc aagagggtct aattacatta aatgaaatat taaattcagg atgtaaggag    120
tcacaactat acattcactt taaagtcaaa tatcgacaaa agctaataga cgtgattaga    180
aaatcacagg cgcaaaaaag aatctgggat aatgcagaga gtattgatgt ttacgaatct    240
gaaaatcaaa ttaattccag taactcaaac cccgaagaca taatagtcta tgacagtctt    300
gtaaaggaag taataacaaa attaacacct tcataccgga aactactgaa acgacatcta    360
agaggtgagg atgtgacaag gatggaaaaa tacagactga aggaacgaat caaacaatt    420
ttatttgatg gtgattga                                438

```

<210> SEQ ID NO 20

<211> LENGTH: 145

<212> TYPE: PRT

<213> ORGANISM: *Lactococcus fujiensis*

<400> SEQUENCE: 20

```

Met Lys Pro Ile Val Ser Lys Ser Met Arg Thr Leu Lys Ile Asn Phe
1      5      10      15
Trp Thr Thr Glu Asp Tyr His Gln Glu Gly Leu Ile Thr Leu Asn Glu
20     25     30
Ile Leu Asn Ser Gly Cys Lys Glu Ser Gln Leu Tyr Ile His Phe Lys
35     40     45
Val Lys Tyr Arg Gln Lys Leu Ile Asp Val Ile Arg Lys Ser Gln Ala
50     55     60
Gln Lys Arg Ile Trp Asp Asn Ala Glu Ser Ile Asp Val Tyr Glu Ser
65     70     75     80
Glu Asn Gln Ile Asn Ser Ser Asn Ser Asn Pro Glu Asp Ile Ile Val
85     90     95
Tyr Asp Ser Leu Val Lys Glu Val Ile Thr Lys Leu Thr Pro Ser Tyr
100    105    110
Arg Lys Leu Leu Lys Arg His Leu Arg Gly Glu Asp Val Thr Arg Met
115    120    125
Glu Lys Tyr Arg Leu Lys Glu Arg Ile Lys Gln Ile Leu Phe Asp Gly
130    135    140
Asp
145

```

<210> SEQ ID NO 21

<211> LENGTH: 480

<212> TYPE: DNA

<213> ORGANISM: *Lactococcus chungangensis*

<400> SEQUENCE: 21

```

atggataaga ttgaaccat acttaaaaat attgaaccga ttatcatgaa ctgtcgaaaa    60
aaaactaaca tcccttctctg gcaattagac gactatctcc aggaaggcat gattattgct    120
ctagagatgt atcatcaact tttattagac ccaccagatg atgacttttaa cttctatggt    180
tatttcaaag tgagatatc ttgtttcttg attgatcagt atcggagaaa catggctgtc    240
aaaagaaaat tcgaccagat tgactattgt gaactatctg aggcgtttta tctttttgat    300

```

-continued

```

caaaatcaag atgtctctga aaacgtcatg tataatttgt tatgtcaaga aatacacttg 360
cttctatctc ctgaagaacg agagcttttt gaggcactta aaaatggaca gaagattgac 420
cgtaatcaaa agtttcgtat caagaagaaa attattgaat atattaagag gttttggtga 480

```

```

<210> SEQ ID NO 22
<211> LENGTH: 159
<212> TYPE: PRT
<213> ORGANISM: Lactococcus chungangensis

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```

<400> SEQUENCE: 22

```

```

Met Asp Lys Ile Glu Thr Ile Leu Lys Asn Ile Glu Pro Ile Ile Met
1           5           10           15

```

```

Asn Cys Arg Lys Lys Thr Asn Ile Pro Ser Trp Gln Leu Asp Asp Tyr
20           25           30

```

```

Leu Gln Glu Gly Met Ile Ile Ala Leu Glu Met Tyr His Gln Leu Leu
35           40           45

```

```

Leu Asp Pro Pro Asp Asp Asp Phe Asn Phe Tyr Val Tyr Phe Lys Val
50           55           60

```

```

Arg Tyr Ser Cys Phe Leu Ile Asp Gln Tyr Arg Arg Asn Met Ala Val
65           70           75           80

```

```

Lys Arg Lys Phe Asp Gln Ile Asp Tyr Cys Glu Leu Ser Glu Ala Phe
85           90           95

```

```

Tyr Leu Phe Asp Gln Asn Gln Asp Val Ser Glu Asn Val Met Tyr Asn
100          105          110

```

```

Leu Leu Cys Gln Glu Ile His Leu Leu Leu Ser Pro Glu Glu Arg Glu
115          120          125

```

```

Leu Phe Glu Ala Leu Lys Asn Gly Gln Lys Ile Asp Arg Asn Gln Lys
130          135          140

```

```

Phe Arg Ile Lys Lys Lys Ile Ile Glu Tyr Ile Lys Arg Phe Trp
145          150          155

```

```

<210> SEQ ID NO 23
<211> LENGTH: 414
<212> TYPE: DNA
<213> ORGANISM: Lactococcus lactis

```

```

<400> SEQUENCE: 23

```

```

atgcctacaa ttaaccaatt ggtacgcaaa cctcgtcgtg ctcaagtgac taaatctaaa 60

```

```

tcaccagcaa tgaacgttgg ctacaacagc cgtaaaaaag tacaaactaa acttgcaagc 120

```

```

ccacaaaaac gtggagtagc aactcgtgtt ggtacaatga ctcctaaaaa acctaactca 180

```

```

gcgcttcgta aattcgcgcg tgtacgtctt tcaaacctta tggaagtaac agcgtacatc 240

```

```

ccaggtatcg gacacaacct ccaagaacac agtgttgtac ttcttcgtgg tggacgtgta 300

```

```

aaagaccttc caggggtacg ttaccatata gtctgtggtg cacttgatac agcaggtgtc 360

```

```

gctgaccgta aacaaagccg ttctaaatac ggtgctaaaa aacaaaaagc ttaa 414

```

```

<210> SEQ ID NO 24
<211> LENGTH: 414
<212> TYPE: DNA
<213> ORGANISM: Lactococcus lactis

```

```

<400> SEQUENCE: 24

```

```

atgcctacaa ttaaccaatt ggtacgcaaa cctcgtcggg ctcaagtgac taaatctaaa 60

```

-continued

tcaccagcaa tgaacgttgg ctacaacagc cgtaaaaaag taaaactaa acttgcaagc	120
ccacaaaaac gtggagtagc aactcgtgtt ggtacaatga ctctataaa acctaactca	180
gcgcttcgta aattcgcgcg tgtacgtctt tcaaacctta tggaagtaac agcgtacatc	240
ccaggtatcg gacacaacct ccaagaacac agtgtgttac ttcttcgtgg tggacgtgta	300
aaagaccttc caggggtacg ttaccatatac gttcgtggtg cacttgatac agcaggtgtc	360
gctgaccgta aacaaagccg ttctaaatac ggtgctaaaa aacaaaaagc ttaa	414

<210> SEQ ID NO 25
 <211> LENGTH: 414
 <212> TYPE: DNA
 <213> ORGANISM: Lactococcus lactis

<400> SEQUENCE: 25

atgcctacaa ttaaccaatt ggtacgcaaa cctcgtcgtg ctcaagtgc taaatctaaa	60
tcaccagcaa tgaacgttgg ctacaacagc cgtaaaaaag taaaactaa acttgcaagc	120
ccacaaaaac gtggagtagc aactcgtgtt ggtactatga ctctaaaaa acctaactca	180
gcgcttcgta aattcgcgcg tgtacgtctt tcaaacctta tggaagtaac agcgtacatc	240
ccaggtatcg gacacaacct ccaagaacac agtgtgttac ttcttcgtgg tggacgtgta	300
aaagaccttc caggggtacg ttaccatatac gttcgtggtg cacttgatac agcaggtgtc	360
gctgaccgta aacaaagccg ttctaaatac ggtgctaaaa aacaaaaagc ttaa	414

<210> SEQ ID NO 26
 <211> LENGTH: 26
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 26

aaaagagctc aattatgaaa aagagg	26
------------------------------	----

<210> SEQ ID NO 27
 <211> LENGTH: 25
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 27

aaaactgcag ttaatcatca tctcg	25
-----------------------------	----

<210> SEQ ID NO 28
 <211> LENGTH: 28
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 28

aaaagagctc ataaaaggag aactttcc	28
--------------------------------	----

<210> SEQ ID NO 29
 <211> LENGTH: 25
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 29

aaaactgcag tcactcttcg tcttc 25

<210> SEQ ID NO 30
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 30

atatcaattg gtcctcggga tatgataag 29

<210> SEQ ID NO 31
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 31

gactttgaac ctcaactcc 19

<210> SEQ ID NO 32
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 32

atagtctcga gtttaagcaa ttgaatcgct ag 32

<210> SEQ ID NO 33
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 33

gcaaaaagtt tccaaatttc atactagaat atacgcaatt tg 42

<210> SEQ ID NO 34
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 34

caaattgcgt atattctagt atgaaatttg gaaacttttt gc 42

<210> SEQ ID NO 35
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 35

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gcgaaaggat ccctattagg tatattccat gtgg 34

<210> SEQ ID NO 36
<211> LENGTH: 37
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 36

gtccctcga gggcggtct gttggattaa tatatgg 37

<210> SEQ ID NO 37
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 37

ctttatgttt ttggcggatc tcatactaga atatacgcaa ttg 44

<210> SEQ ID NO 38
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 38

caaatgcgt atattctagt atgagatccg ccaaaaacat aaag 44

<210> SEQ ID NO 39
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 39

gcgaaaggat ccttacaatt tgggctttcc g 31

<210> SEQ ID NO 40
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 40

aaaaccggg tttaagcaat tgaatcgta g 31

<210> SEQ ID NO 41
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 41

aaaaccggg aaataaatgg ctacaaaatt 30

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<210> SEQ ID NO 42
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 42

aaaacggcgcg ttacaatttg ggctttccg 29

<210> SEQ ID NO 43
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 43

atagtctcga gaaataaatg gctacaaaat t 31

<210> SEQ ID NO 44
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 44

ctttatgttt ttggcggatc tcatactaga ctatacgcaa ataatc 46

<210> SEQ ID NO 45
<211> LENGTH: 46
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 45

gattatttgc gtatagtcta gtatgagatc cgccaaaaac ataaag 46

<210> SEQ ID NO 46
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 46

agcttcctaa tacaacacaa ttaatatgt gttgtattat tg 42

<210> SEQ ID NO 47
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 47

aattcaataa tacaacacaa tattaattgt gttgtattag ga 42

<210> SEQ ID NO 48
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 48

atgcctacaa ttaaccaat 19

<210> SEQ ID NO 49
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 49

caccgtatattt agaacgg 17

<210> SEQ ID NO 50
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 50

atgcctacta ttaaccaat 19

<210> SEQ ID NO 51
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 51

taccgtatattt agaacgg 17

<210> SEQ ID NO 52
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 52

agtagtatca gcactgacag c 21

<210> SEQ ID NO 53
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 53

acacctttgt tcttgaagg 19

<210> SEQ ID NO 54
<211> LENGTH: 30
<212> TYPE: DNA
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<400> SEQUENCE: 57

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<212> TYPE: DNA
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<400> SEQUENCE: 58

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<400> SEQUENCE: 59

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<400> SEQUENCE: 61

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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 62

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<210> SEQ ID NO 63
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 63

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<210> SEQ ID NO 64
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 64

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<400> SEQUENCE: 65

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<210> SEQ ID NO 66
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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 66

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<400> SEQUENCE: 73

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<400> SEQUENCE: 74

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<400> SEQUENCE: 76

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<210> SEQ ID NO 77
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<400> SEQUENCE: 77

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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 78

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<212> TYPE: DNA
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<400> SEQUENCE: 80

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<400> SEQUENCE: 81

ttgctttaag gatagtttc 19

<210> SEQ ID NO 82
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<400> SEQUENCE: 82

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<210> SEQ ID NO 83
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<212> TYPE: DNA
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<400> SEQUENCE: 83

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<210> SEQ ID NO 85
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

<400> SEQUENCE: 85

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<210> SEQ ID NO 86
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<212> TYPE: DNA
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<220> FEATURE:
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<220> FEATURE:
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<210> SEQ ID NO 88
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<212> TYPE: DNA
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<210> SEQ ID NO 89
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<220> FEATURE:
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<210> SEQ ID NO 90
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<212> TYPE: DNA
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<220> FEATURE:
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<223> OTHER INFORMATION: Oligonucleotide primer

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<400> SEQUENCE: 92

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<210> SEQ ID NO 93
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<210> SEQ ID NO 94
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<400> SEQUENCE: 95

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<210> SEQ ID NO 96
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<223> OTHER INFORMATION: Oligonucleotide primer

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<400> SEQUENCE: 96

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1. A method for transforming a strain of the *Lactococcus* genus with an exogenous DNA polynucleotide comprising the steps of:

- (a) providing a strain of the *Lactococcus* genus, wherein said strain is transformable through natural competence;
- (b) modulating the production of a ComX protein in said strain;
- (c) contacting said strain of step (b) with an exogenous DNA polynucleotide in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and
- (d) selecting a strain which has integrated the exogenous DNA polynucleotide into its genome.

2. A method according to claim 1, wherein the step of modulating the production of a ComX protein is performed by expressing a comX gene in said strain or increasing the expression of a comX gene in said strain.

3. A method according to claim 2, wherein said comX gene is an exogenous comX gene.

4. A method according to claim 3, wherein said exogenous comX gene is transferred into said strain by conjugation, transduction, or transformation.

5. A method according to claim 2, wherein said comX gene is the endogenous comX gene of said strain.

6. A method according to claim 5, wherein the method comprises carrying out step (b) and then carrying out step (c) or comprises carrying out step (b) and step (c) simultaneously.

7. A method according to claim 1, wherein said ComX protein has:

the amino acid sequence of SEQ ID NO: 2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20 or SEQ ID NO:22;

an amino acid sequence having at least 90% identity to the amino acid sequence of SEQ ID NO: 2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID

NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20 or SEQ ID NO:22; or an amino acid sequence having at least 90% similarity to the amino acid sequence of SEQ ID NO: 2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20 or SEQ ID NO:22.

8. A method according to claim 1, wherein said comX gene has:

the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, or SEQ ID NO:21; or

a nucleotide sequence having at least 90% identity to the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, or SEQ ID NO:21.

9. A method according to claim 1, wherein said medium of step (c) is a chemically defined medium.

10. A method according to claim 1, wherein prior to step (c) said strain is incubated in a pre-culture medium.

11. A method according to claim 1, wherein said strain is incubated with the exogenous DNA polynucleotide for around 4-8 hours at around 30° C. and said medium of step (c) is supplemented with an osmo-stabilizer.

12. A method according to claim 1, wherein said strain of the *Lactococcus* genus of step (a) is a strain of the *Lactococcus raffinolactis* species or a strain of the *Lactococcus lactis* species.

13. A method according to claim 1, wherein said exogenous DNA polynucleotide used in step (c) is obtained from a strain of the same species as the strain provided in step (a).

14. A strain of the *Lactococcus* genus obtained by the method of claim 1.

15. A method for identifying a strain of the *Lactococcus* genus which is transformable through natural competence comprising the steps of:

- (a) providing a strain of the *Lactococcus* genus;
- (b) transforming said strain with a plasmid expressing a comX gene having at least 90% identity to the endogenous comX gene of said strain;
- (c) contacting said strain obtained in step (b) with an exogenous DNA polynucleotide encoding a marker gene in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and
- (d) determining the rate of recombination events; wherein a rate of at least 1×10^{-6} transformants per μg of DNA is indicative of a strain which is transformable through natural competence.

16. A method according claim 1, wherein said strain of step (a) is identified using a method for identifying a strain of the *Lactococcus* genus which is transformable through natural competence comprising:

- (a) providing a strain of the *Lactococcus* genus;
- (b) transforming said strain with a plasmid expressing a comX gene having at least 90% identity to the endogenous comX gene of said strain;
- (c) contacting said strain obtained in step (b) with an exogenous DNA polynucleotide encoding a marker gene in a medium and incubating the resulting mixture for integration of the exogenous DNA polynucleotide into the genome of said strain; and
- (d) determining the rate of recombination events; wherein a rate of at least 1×10^{-6} transformants per μg of DNA is indicative of a strain which is transformable through natural competence.

17. A method according to claim 1, wherein said strain of step (a) is identified using assay A, which is performed as follows:

- i) providing a strain of the *Lactococcus* genus;
- ii) transforming the strain with a plasmid expressing a comX gene having at least 90% identity to the endogenous comX gene of the strain;
- iii) pre-culturing the transformed strain overnight in a complex medium supplemented with glucose;
- iv) diluting about 1.5 mL of the pre-culture in 8.5 mL of fresh medium;
- v) after 2 hr of further growth at 30° C., washing the cells twice with distilled water and adjusting the OD₆₀₀ to 0.05 in a chemically defined medium comprising 5 $\mu\text{g mL}^{-1}$ erythromycin and an osmo-stabilizer;
- vi) adding 5 μg of exogenous DNA polynucleotide bearing an antibiotic resistance gene to 300 μL of the culture medium;
- vii) incubating the resulting culture for 6 hr at 30° C.;
- viii) plating the cells onto agar plates comprising the complex medium supplemented with glucose and antibiotic corresponding to the antibiotic resistance gene of the exogenous DNA polynucleotide and incubating for 48 hr;
- ix) counting the colony forming units and determining the transformation rate, wherein:
 - the transformation rate equals the number of antibiotic-resistance colony forming units per mL divided by the total number of viable colony forming units per mL, and
 - a transformation rate of at least 1×10^{-6} transformants per μg of DNA is indicative of a strain that is transformable through natural competence.

18. A method according to claim 1, wherein said ComX protein has the amino acid sequence of SEQ ID NO: 2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20 or SEQ ID NO:22.

19. A method according to claim 1, wherein said comX gene has the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, or SEQ ID NO:21.

20. A method according to claim 11, wherein the osmo-stabilizer is glycerol or mannitol.

* * * * *