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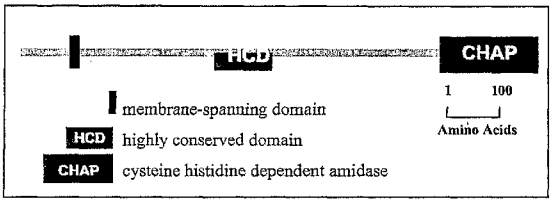
(54) **Title**: NOVEL BACTERIAL CELLS AND USES THEREOF

FIGURE 4



(57) **Abstract**: The present invention provides a bacterial host cell having improved cell permeability properties, the cell comprising an Orf18 gene or species homologue thereof, or a fragment or variant of the same encoding a polypeptide having the activity of the Orf18 gene product or species homologue thereof, wherein the gene, homologue, fragment or variant is under the control of an heterologous promoter (inducible or constitutively active) which permits sufficient expression of the gene, homologue, fragment or variant to increase the permeability of the cell wall. "Orf18 and the "orf18 gene" are also known as "CsiA" and the "csiA gene" respectively. In one embodiment, the host cell is a *Lactococcus lactis* cell in which a chromosomally-integrated Orf18 gene naturally present in the cell is inactivated. The invention further provides the use of such host cells in the production of polypeptides. In addition, the invention provides pharmaceutical compositions of the host cells and the use thereof for administering a bioactive agent to the human or animal body (for example, to the GI tract).

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NOVEL BACTERIAL CELLS AND USES THEREOF

Field of Invention

The present invention relates to bacterial host cells having improved cell permeability properties, which properties may be constitutive or inducible. Such host cells are useful in the production of polypeptides, as protected delivery vehicles for the site-specific administration of bioactive agents within the body and for use in industrial fermentation (for example, for accelerated flavour development for matured cheeses in the dairy industry).

Background

Recombinant DNA methods make it feasible to clone specific DNA fragments from any source into vectors that can be studied in well-characterized bacteria and eukaryotic cells. Applications of DNA cloning are expanding rapidly in all fields of biology and medicine. Pharmaceutical applications include large-scale production from cloned human genes of biologic products with therapeutic value, such as polypeptide hormones, interleukins, and enzymes. Applications in public health and laboratory medicine include development of vaccines to prevent specific infections and probes to diagnose specific infections by nucleic acid hybridization or polymerase chain reaction (PCR). Industrial applications include the production of enzymes and other proteins for use in both manufactured foods and in non-food applications.

A number of different expression systems are available for the production of recombinant proteins, including the use of prokaryotic hosts (including bacteria such as *E. coli*, *Lactococcus* and *Bacillus*) and eukaryotic hosts (including yeast cells, mammalian cells and insect cells) system. Of the available recombinant protein expression systems, prokaryotic systems have several advantages; these include ease of culture, rapid cell growth and ease of induction of bacterial protein expression.

After expression of the gene product, purification of the protein is required. Since the cloned gene is expressed in a host cell, the protein of interest must be purified from the endogenous proteins of that host cell. Various approaches have been utilised to aid this purification process. One option is to tag the cloned gene product, for example with a histidine (His) tag or any other marker protein which facilitates purification of the expressed protein. Alternatively, the cloned gene may be modified to include in signal sequence, such that the expressed fusion protein comprises a signal peptide to direct exportation of the protein from the cell. In a further alternative approach, external lytic agents are applied to lyse the cells. However, such approaches have the potential to complicate and disrupt polypeptide expression.

However, there remains a need for improved recombinant protein expression systems which permit easy purification.

Summary of Invention

The first aspect of the invention provides a bacterial host cell having improved cell permeability properties, the cell comprising an *orf18* gene or species homologue thereof, or a fragment or variant of the same encoding a polypeptide having the activity of the *orf18* gene product or species homologue thereof, wherein the gene, homologue, fragment or variant is under the control of an heterologous promoter which permits sufficient expression of the gene, homologue, fragment or variant to increase the permeability of the cell wall.

"Orf18" and the "*orf18*" gene are also known as "CsiA" and the "*csiA* gene" respectively.

By "*orf18* gene" we mean the *orf18* gene present in a transmissible genetic element (the sex factor) that is integrated within the chromosome of *Lactococcus lactis* bacteria, such as *Lactococcus lactis* MG1363. In a preferred embodiment, the *orf18* gene comprises a nucleotide sequence as shown in Figure 3 [SEQ ID NO: 1]. It will be appreciated that the *orf18* gene may alternatively comprise a naturally occurring variant of the nucleotide sequence of SEQ ID NO: 1.

By "improved cell permeability properties" we mean the wall and/or membrane of the bacterial host cell exhibits enhanced permeability to macromolecules (*e.g.* polypeptides)

relative to the cell wall and/or membrane permeability of an equivalent cell in which the *orf18* gene, homologue, fragment or variant is not expressed, or that such enhanced permeability is capable of being induced in the host cell. It will be appreciated that the enhanced permeability of the cell may increase the release of polypeptides from the host cell.

In one embodiment, the cell is incapable of inducible cell lysis.

In an alternative embodiment, the cell is capable of inducible cell lysis. Release of large macromolecules such as genomic DNA may be indicative of cell lysis rather than cell leakage. Thus, the *orf18* gene, homologue, fragment or variant may be under the control of an inducible promoter.

In a further embodiment, the cell wall is more permeable than a corresponding host cell lacking the *orf18* gene, homologue, fragment or variant thereof. Thus, the *orf18* gene, homologue, fragment or variant may be under the control of a constitutive promoter of suitable strength to render the cell wall more permeable whilst still retaining cell viability.

By "species homologue" we mean a naturally occurring homologue of the *orf18* gene of *Lactococcus lactis* MG1363, which homologue retains the cell lytic property of the *orf18* gene product.

By the "activity of the *orf18* gene product or species homologue thereof" we mean the ability of the *orf18* gene product or species homologue thereof, respectively, to increase the permeability of the cell wall and/or to induce lysis of the bacterial cell. Such activity may be determined using methods well known in the art (see Examples below). It is believed that the *orf18* gene product is able to increase the permeability of the cell wall by inhibiting bacterial cell wall synthesis, by blocking the production of and/or breaking cross-links in cell wall peptidoglycans.

By "an heterologous promoter" we include a promoter other than the wildtype promoter controlling the expression of the *orf18* gene or species homologue thereof in naturally-occurring bacterial cells. However, where the bacterial cell does not naturally contain an *orf18* gene or species homologue thereof, it will be appreciated that any promoter may be regarded as an heterologous promoter in respect of that cell. Thus, the invention encompasses host cells not naturally containing an *orf18* gene or species homologue

thereof, which have been engineered to contain an *orf18* gene, homologue, fragment or variant thereof under the control of any suitable promoter (including a promoter which is known to be associated with an *orf18* gene or species homologue thereof in a different species/strain of bacterial cell).

The host cells may be isolated and provided in the form of a culture of cells.

In one embodiment, the bacterial host cell comprises (or formerly comprised) a chromosomally-integrated *orf18* gene naturally present in the bacterial cell is inactivated. For example, the chromosomally-integrated *orf18* gene naturally present in the bacterial cell may be deleted, in whole or in part, such that expression of a functionally active gene product is prevented.

It will be appreciated that any suitable bacterial host cell may be used in which the phenotype of Orf18 can be expressed, for example a (non-pathogenic) Gram-positive bacterial cell (the DNA of which may be AT-rich). Thus, the host cell may be selected from the group consisting of *Lactococcus* cells, *Lactobacillus* cells, *Bacillus subtilis* cells and *Clostridium* cells.

Preferably, the host cell is a Gram-positive cocci. More preferably the host cell is a Gram-positive cocci which has *orf18* gene homologue(s) in its conjugation system(s). Preferably the host cell is selected from the group consisting of *Enterococcus* cells and *Streptococcus* cells. More preferably, the host cell is selected from the group consisting of *Enterococcus faecalis*, *Streptococcus agalactiae* and *Streptococcus suis*. Alternatively, a (non-pathogenic) Gram-negative bacterial cell may be used, for example *Escherichia coli.*

In one embodiment, the cell is a *Lactococcus* cell. Suitable *Lactococcus* cells are available from the NCIMB culture collection and include (but are not limited to) the following:

NCIMB8662 (=HP)
NCIMB700499 (=UD459)
NCIMB700500 (=UD496)
NCIMB700504 (=UD806)
NCIMB700508 (=C7)

NCIMB700562 (=D31)
NCIMB700609 (=RW)
NCIMB700762 (=ML1)
NCIMB70216 (=SK11)
NCIMB700278 (=FD50)
NCIMB7012008 (=TR)

Cells of *Lactococcus lactis* are especially suited to the present invention. For example, the cell may be a *Lactococcus lactis* cell of strain MG1363.

In an alternative embodiment, the cell is a *Lactobacillus* cell (for example *Lactobacillus johnsonii* FI9785).

In a further alternative embodiment, the cell is a *Bacillus subtilis* cell (for example *Bacillus subtilis* 168).

It will be appreciated by persons skilled in the art that the *orf18* gene, or a homologue from another *Lactococcus* strain or another bacterial species, a fragment or variant thereof may be located extra-chromosomally on a plasmid. Suitable plasmids include but are not limited to pUK200, LP712, pAMβ1 and pIL501. It will be further appreciated by skilled persons that derived vectors which include the replicons of such plasmids may also be used, in addition to the plasmids themselves.

Alternatively, the *orf18* gene, species homologue, fragment or variant thereof may be chromosomally integrated.

Methods suitable for the transformation of bacterial cells with a plasmid and chromosomal integration of a polynucleotide construct are well known in the art (for example, see *Molecular Cloning: a Laboratory Manual*, 3rd edition, Sambrook & Russell, 2001, Cold Spring Harbor Laboratory Press, the disclosures of which are incorporated herein by reference). For example, Lactococcal cells may be transformed using the electroporation technique described in Holo, H & Nes, I. F. (1989) High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilised media. *Applied and Environmental Microbiology* **55**: 3119-3123 (the disclosures of which are incorporated herein by reference).

An exemplary protocol for transforming *Lactococcus lactis* cells with a plasmid is described below (see Examples).

Where a bacterial cell naturally contains an *orf18* gene or species homologue thereof, a host cell according to the invention may be produced by *in vivo* rearrangement (without the need for genetic manipulation by recombinant methodologies). Methods for producing such *in vivo* rearrangement are well known in the art, for example see Gasson *et al.*, 1992, *Mol. Microbiol.* **6**(21):3213-3223 (the disclosures of which are incorporated herein by reference).

Thus, in one embodiment of the first aspect of the invention, the host cells comprise a naturally-occurring chromosomal *orf18* gene, or homologue thereof, under the control of an heterologous promoter.

In one embodiment of the first aspect of the invention, the host cell comprises a complete *orf18* gene. The *orf18* gene may comprise or consist of the nucleotide sequence of SEQ ID NO:1.

In an alternative embodiment of the first aspect of the invention, the host cell comprises a fragment of an *orf18* gene.

The fragment may comprise or consist of at least 100 contiguous nucleotides of SEQ ID NO: 1, for example at least 200, 300, 500, 1000, 1500, 2000 or 2500 contiguous nucleotides of SEQ ID NO: 1.

The *orf18* gene fragment must encode a polypeptide which retains the cell lytic activity of the full-length *orf18* gene product.

As discussed below in the Examples, it has been found that the *orf18* gene contains a distinct domain of 95amino acids (previously thought to be 130 amino acids) which is highly conserved between six species homologues; this domain is termed the 'highly conserved domain' or HCD. Point mutations in the HCD render the Orf18 polypeptide incapable of inducing cell lysis. Within this domain, the role of amino acid R577 in cell lysis appears to be of particular importance.

Accordingly, the *orf18* gene fragment preferably comprises amino acid R577 and, more preferably, the entire HCD.

In contrast to the HCD, the cysteine, histidine-dependent aminohydrolase/peptidase (CHAP) domain of the *orf18* gene has been found not to be necessary for cell lysis.

Accordingly, in an alternative embodiment, the *orf18* gene fragment excludes a C-terminal cysteine, histidine-dependent aminohydrolase/peptidase domain (CHAP) domain of the *orf18* gene.

In a further alternative embodiment of the first aspect of the invention, the host cell comprises a variant of an *orf18* gene, or variant of a fragment thereof, under the control of an inducible promoter.

By 'variant' we include nucleic acid molecules which contain insertions, deletions and/or substitutions, either conservative or non-conservative, relative to the nucleotide sequence of SEQ ID NO:1. In particular, the variant may be a non-naturally occurring variant.

For example, the variant may comprise a nucleotide sequence with at least 60% identity to the nucleotide sequence of SEQ ID NO: 1, more preferably at least 70% or 80% or 85% or 90% identity to said sequence, and most preferably at least 95%, 96%, 97%, 98% or 99% identity to said amino acid sequence.

It will be appreciated that the above sequence identity may be over the full length of the nucleotide sequence of SEQ ID NO: 1 or over a portion thereof. Preferably, however, the sequence identity is over at least 100 nucleotides of the nucleotide sequence of SEQ ID NO:1, for example at least 200, 300, 500, 1000, 1500, 2000 or 2500 more nucleotides therein.

Percent identity can be determined by methods well known in the art, for example using the LALIGN program (Huang and Miller, Adv. Appl. Math. (1991) 12:337-357, the disclosures of which are incorporated herein by reference) at the Expasy facility website:

www.ch.embnet.org/software/LALIGN_form.html

using as parameters the global alignment option, scoring matrix BLOSUM62, opening gap penalty –14, extending gap penalty –4.

Alternatively, the percent sequence identity between two nucleotide sequences may be determined using suitable computer programs, for example AlignX, Vector NTI Advance 10 (from Invitrogen Corporation) or the GAP program (from the University of Wisconsin Genetic Computing Group).

It will be appreciated that percent identity is calculated in relation to polynucleotides whose sequence has been aligned optimally.

Fragments and variants of the amino acid sequence of SEQ ID NO: 1 may be made using the methods of gene engineering and site-directed mutagenesis well known in the art (for example, see *Molecular Cloning: a Laboratory Manual*, 3rd edition, Sambrook & Russell, 2001, Cold Spring Harbor Laboratory Press, the disclosures of which are incorporated herein by reference).

In one preferred embodiment of the host cells of the invention, the *orf18* gene, homologue, fragment or variant is under the control of an inducible promoter. The inducibility of the promoter permits control of the rate of leakage of polypeptides from the cell and, ultimately, cell lysis.

Suitable inducible promoters are well known to those skilled in the art. For example, the inducible promoter may be P_{nisA}.

It will be appreciated that other inducible expression systems may also be utilised. For example, a promoter capable of being induced by a suitable carbon source may be used, such as the xylose inducible expression system for *Lactococcus lactis* (see Miyoshi *et al.*, 2004, *FEMS Microbiology Letters* **70** (9): 5398-5406). Similar lactose- and/or sucrose-inducible expression systems also exist. However, such systems may need to be engineered to ensure that the promoter is sufficiently inactive in the absence of the carbon source inducer.

Alternatively, a late/stationary phase promoter may be used. Such promoters, and methods for their identification, are well known in the art. For example, see Hengge-Aronis, 1996, Regulation of gene expression during entry into stationary phase, p. 1497–

1512. In "*Escherichia coli and Salmonella: cellular and molecular biology*", Neidhardt *et al.* (eds.), 2nd ed. ASM Press, Washington, D.C; Tomohiro Shimada *et al.*, 2004, *J Bacteriol.* **186**(21): 7112–7122; European Patent No. 1 244 799 B; Israelsen et al., 1995, *Appl Environ Microbiol.* **61**(7): 2540–2547; Martín *et al.*, 2004, *J Bacteriol.* **186**(17): 5649–5660; Kilstrup *et al.*, 1997, *Appl Environ Microbiol.* **63**(5): 1826–1837, US Patent No. 7,125,690 (the disclosures of which are incorporated herein by reference).

In an alternative preferred embodiment of the host cells of the invention, the *orf18* gene, homologue, fragment or variant is under the control of a constitutive promoter. The strength of the constitutive promoter should allow sufficient expression of the *orf18* gene, homologue, fragment or variant to improve the permeability of the host cell wall (in turn, allowing increased release of polypeptides from the host cell). It will be appreciated that the constitutive promoter should not allow sufficient expression of the *orf18* gene, homologue, fragment or variant to lyse the cells. Thus, the host cells should preferably remain viable.

Constitutive promoters having a wide range of different strengths are well known in the art (for example, see Jensen & Hammer, 1998, *Applied Env. Microbiol.* **64**(1):82-87 and Jensen & Hammer, 1998, *Biotech. Bioeng.* **58**(2-3):191-195). Skilled persons would readily be able to select from these known promoters a constitutive promoter having the desired strength. For example, a promoter probe approach may be used to isolate a pre-existing promoter that reproduces the sought after phenotype (cell 'leakiness'). For this, one would have constitutive intracellular expression of a marker gene the product of which is detectable after its release from the cell (*e.g.* amylase). A promoterless *orf18* gene (perhaps linked to a promoterless selection marker such as an antibiotic resistance gene, *e.g.* chloramphenicol resistance) could then be used as a promoter probe vector with random inserts placed upstream and surviving (selected chloramphenicol resistant) colonies screened for release of the marker (*e.g.* amylase enzyme activity as a colorimetric test).

The enhanced cell wall permeability properties of the host cells of the invention make them ideally suited for use in the production of polypeptides. Controlled release of polypeptides from the cells can be achieved without the need for signal peptides or external lytic agents, which can complicate polypeptide expression and purification.

Thus, in a further embodiment of the first aspect of the invention, the host cell further comprises a polypeptide for release and/or a nucleic acid molecule encoding the same.

It will be appreciated that the host cells of the invention are suitable for use in the production of any polypeptide that may be expressed in a bacterial cell.

In one embodiment, the polypeptide for release is a bioactive polypeptide, including bioactive polypeptides selected from the group consisting of vaccine antigens, immune modulators, antimicrobial agents, anti-angiogenic agents and growth factors.

For example, the polypeptide for release may be an interleukin, such as interleukin-10 or interleukin-12.

The bioactive polypeptide may have efficacy in the treatment of inflammatory bowel disorder, for example interleukin-10, keratinocyte growth factor (KGF), trefoil factor (TFF) or transforming growth factor (TGF)-β.

Alternatively, the bioactive polypeptide may have efficacy in the treatment of colon cancer, for example endostatin or soluble vascular endothelial growth factor receptor VEGFR-1.

It will be appreciated that the polypeptide for release may be a polypeptide for industrial (*e.g.* pharmaceutical) or domestic use, such as an enzyme. Such polypeptides include peptidases, proteinases, esterases, lipases and endolysins.

In one embodiment, the polypeptide for release is an endolysin, such as an endolysin selected from a group consisting of endolysins that target *Streptococcus pneumoniae* (for example Pal, Cpl-1 and LytA), endolysins that target *Clostridium perfringens* (for example, the lysin of bacteriophage ϕ3626), endolysins that target *Bacillus anthracis* / *Bacillus cereus* (for example, endolysin PlyG), endolysins that target *Staphylococcus aureus* (for example, endolysins phi11 and phi 12) and endolysins that target *Enterococcus faecalis* and *faecium* (for example, endolysin PlyV12).

In one preferred embodiment, the polypeptide for release is a bacteriophage endolysin, such as an endolysin of a bacteriophage selected from the group consisting of

bacteriophage ΦCD27 of *Clostridium difficile*, bacteriophage ΦP1 of *Clostridium tyrobutyricum* and bacteriophage ΦLM4 of *Listeria monocytogenes.*

Such endolysins are described in Hermoso *et al.* (2007) "Taking aim on bacterial pathogens: from phage therapy to enzybiotics". *Current Opinion in Microbiology* **10**: 461-472 (the disclosures of which are incorporated herein by reference).

As discussed above, in one embodiment of the first aspect of the invention the host cell comprises a species homologue of an *orf18* gene, or a fragment or variant of the same encoding a polypeptide having the activity of the *orf18* gene product or species homologue thereof. For example, the species homologue may be selected from the group consisting of the *B0020* gene of conjugative plasmid pTEF2 of *Enterococcus faecalis*, the *prgK* gene of conjugative plasmid pCF10 of *Enterococcus faecalis*, the *SAG1286* gene of conjugative transposon Tn5252 of *Streptococcus agalactiae* and the *gbs1133* and *gbs1359* genes of *Streptococcus agalactiae* strain NEM316.

A second aspect of the invention provides a kit for use in the production of a recombinant protein, the kit comprising:

(a) a bacterial cell capable of exhibiting improved cell permeability properties upon transformation with a plasmid comprising an *orf18* gene or species homologue thereof, or a fragment or variant of the same encoding a polypeptide having the activity of the *orf18* gene product or species homologue thereof, wherein the gene, homologue, fragment or variant is under the control of an heterologous promoter which permits sufficient expression of the gene, homologue, fragment or variant to increase the permeability of the cell; and

(b) a plasmid comprising an *orf18* gene or species homologue thereof, or a fragment or variant of the same encoding a polypeptide having the activity of the *orf18* gene product or species homologue thereof, wherein the gene, homologue, fragment or variant is under the control of an heterologous promoter which permits sufficient expression of the gene, homologue, fragment or variant to increase the permeability of the cell.

Thus, the kit is suitable for making a host cell according to the first aspect of the invention

In one embodiment, the bacterial cell comprises (or formerly comprised) a chromosomally-integrated *orf18* gene naturally present in the bacterial cell which is inactivated. For example, the chromosomally-integrated *orf18* gene naturally present in the bacterial cell may be deleted, in whole or in part, such that expression of a functionally active gene product is prevented.

It will be appreciated that any suitable bacterial host cell may be used in which the phenotype of Orf18 can be expressed, for example AT-rich Gram-positive bacterial cells. Thus, the cell may be selected from the group consisting of *Lactococcus* cells, *Lactobacillus* cells, *Bacillus subtilis* cells and *Clostridium* cells. Alternatively, a (non-pathogenic) Gram-negative bacterial cell may be used, for example *Escherichia coli*.

In one embodiment, the cell is a *Lactococcus* cell (see above). For example, a *Lactococcus lactis* cell may be used, such as strain MG1363.

It will be appreciated by persons skilled in the art that the plasmid component of the kit may be separate from or contained within the bacterial cell. Suitable plasmids include but are not limited to pUK200, LP712, pAMβ1 and pIL501.

The *orf18* gene or species homologue, or fragment or variant thereof, may be a gene, homologue, fragment or variant as defined above in relation to the first aspect of the invention.

Thus, in one embodiment, the kit comprises a bacterial host cell according to the first aspect of the invention.

The kit may further comprise an inducer for inducing expression of the *orf18* gene, homologue, fragment or variant thereof. For example, when the inducible promoter controlling expression of the *orf18* gene is P_{nisA}, the inducer nisin may be included.

The kit may additionally comprise one or more reagents or media for cell culture.

It will be appreciated that the kits of the invention should be sealed prior to use to prevent contamination.

A third aspect of the invention provides an isolated nucleic acid molecule comprising an *orf18* gene or species homologue thereof, or a fragment or variant of the same encoding a polypeptide having the activity of the *orf18* gene product or species homologue thereof, wherein the gene, homologue, fragment or variant is under the control of an heterologous promoter which permits sufficient expression of the gene, homologue, fragment or variant to increase the permeability of the cell. The *orf18* gene or species homologue, or fragment or variant thereof, may be a gene, homologue, fragment or variant as defined above in relation to the first aspect of the invention.

The isolated nucleic acid molecule may comprise or consist of DNA (such as complementary DNA or genomic DNA) or RNA. In one embodiment, the isolated nucleic acid molecule comprises or consists of DNA, for example complementary DNA.

Conveniently, the isolated nucleic acid molecule is provided in the form of a plasmid.

A fourth aspect of the invention provides the use of a bacterial host cell according to the first aspect of the invention in the production of a polypeptide, which may be endogenous to the host cell or expressed therein by recombinant means.

A related, fifth aspect of the invention provides a method for producing a polypeptide comprising culturing a host cell according to the first aspect of the invention under conditions which allow expression of the polypeptide for release.

Where the *orf18* gene, homologue, fragment or variant thereof is under the control of an inducible promoter, the method may further comprise permitting or enhancing release of the polypeptide from the host cells by exposing the cells to an inducer for inducing expression of the *orf18* gene, homologue, fragment or variant thereof. In one embodiment, the inducer is used in a concentration sufficient to lyse the cells. However, the inducer may alternatively be used in a concentration sufficient to permit or enhance release of the polypeptide from the host cells but not enough to lyse the cells.

Where the *orf18* gene, homologue, fragment or variant thereof is under the control of a constitutive promoter, the promoter is of suitable strength to render the cells 'leaky' (*i.e.* increased cell wall permeability to polypeptides) whilst still retaining their viability.

The released polypeptide may be further purified using methods well known in the art (for example see *Protein Purification: Principles and Practice*, 1993, Robert K Scopes (Ed.), Springer and *Protein Purification Techniques: A Practical Approach*, 2001, Simon Roe (Ed.), Oxford University Press, the disclosures of which is incorporated herein by reference).

In one embodiment, a simple purification step is used in which the cell culture is centrifuged to remove the cells and the released polypeptide obtained from the resulting supernatant.

A sixth aspect of the invention provides a pharmaceutical composition comprising a bacterial host cell according to the first aspect of the invention.

The host cells may be formulated at various concentrations, depending on the efficacy/toxicity of the polypeptide to be released.

It will be appreciated by persons skilled in the art that the host cells of the invention are generally administered in admixture with a suitable pharmaceutical excipient, diluent or carrier selected with regard to the intended route of administration and standard pharmaceutical practice (for example, see *Remington: The Science and Practice of Pharmacy*, 19th edition, 1995, Ed. Alfonso Gennaro, Mack Publishing Company, Pennsylvania, USA, the relevant disclosures in which document are hereby incorporated by reference).

For example, the host cells can be administered orally in the form of tablets, capsules, ovules, elixirs, solutions or suspensions, which may contain flavouring or colouring agents, for immediate-, delayed- or controlled-release applications.

Thus, in one embodiment, the host cells and pharmaceutical compositions thereof are for oral administration. For example, the pharmaceutical composition may be a liquid or capsule containing a liquid.

A seventh aspect of the invention provides a host cell according to the first aspect of the invention for use in medicine.

In one embodiment, the host cell is for administering a bioactive agent to the human or animal body. For example, the host cell may be particularly suited for delivering a bioactive agent to the gastrointestinal (GI) tract. Alternatively, the host cell may be particularly suited for delivering a bioactive agent to the vagina.

By "bioactive agent" we mean any agent capable of being delivered in a bacterial cell which has utility in the therapy, prophylaxis, diagnosis and/or prognosis of a disease or condition of the human or animal body. For example, the agent may be a therapeutic polypeptide.

An eighth aspect of the invention provides the use of a host cell according to the first aspect of the invention in the manufacture of a medicament for administering a bioactive agent to the human or animal body. For example, the medicament may be useful for delivering a bioactive agent to the GI tract.

A ninth, related aspect of the invention provides a method for administering a bioactive agent to the human or animal body comprising administering a host cell according to the first aspect of the invention or a pharmaceutical composition according to the sixth aspect of the invention.

Host cells in which the *orf18* gene, species homologue, fragment or variant is under the control of an inducible promoter are particularly suited to such a method. For example, where a P_{nisA} inducible promoter is used to control expression of the *orf18* gene, species homologue, fragment or variant, administration of a host cell of the invention may be followed after a suitable time interval by administration of a nisin-producing *Lactococcus* cell; availability of the nisin would then induce expression of the *orf18* gene, species homologue, fragment or variant at the desired location within the GI tract. Alternatively, encapsulated nisin may be used (in which the nisin is protected from proteolysis as it passes through the GI tract). In a further alternative, dead host cells of the invention could be used, for example after receiving a lethal heat or radiation treatment.

Where a carbon source-induced promoter is used, such as the xylose-inducible expression system for *Lactococcus lactis*, the appropriate carbon-source may be fed to the patient to induce expression of the *orf18* gene, species homologue, fragment or variant at the desired location within the GI tract.

In one embodiment, the method is for administering a bioactive agent to the GI tract.

It will be appreciated by persons skilled in the art that the bacterial host cells of the invention also have utility outside medicine, for example in industrial or domestic settings.

For example, a tenth aspect of the invention provides the use of a bacterial host cell according to the first aspect of the invention in the production of a dairy product (such as cheese).

Thus, an eleventh aspect of the invention provides a method for producing a dairy product comprising exposing milk to a starter culture of host cells according to the first aspect of the invention, wherein the host cells are lactic acid bacterial cells.

Where the *orf18* gene, homologue, fragment or variant thereof is under the control of an inducible promoter, the method may further comprise exposing the bacterial cells to an inducer to induce cell lysis (for example, after primary fermentation).

A twelfth aspect of the invention provides a method of transforming a host cell according to the first aspect of the invention comprising exposing the cell to an inducer of expression of the *orf18* gene or species homologue thereof, or a fragment or variant of the same, wherein the concentration of inducer is sufficient to inhibit cell wall synthesis but not enough to lyse the cells.

It will be appreciated that host cells in which the *orf18* gene is under the control of a constitutive promoter may also be useful in such methods of transformations.
In one embodiment, the cells are osmotically buffered.

In a further embodiment, the method comprises electroporation of the cells.

Preferred, non-limiting examples which embody certain aspects of the invention will now be described, with reference to the following figures:

Figure 1: Diagram of the lactococcal sex factor based on the genome sequence of *Lactococcus lactis* MG1363

See also Wegmann *et al.*, 2007, *J. Bacteriol.* **189**:3256-3270 [8].

Figure 2: Comparison of the sex factor *cluA-orf22* region with the equivalent regions in *Enterococcus* and *Streptococcus*.

The sex factor of *L. lactis* shares homology with five ancestrally related Gram-positive conjugation systems. A schematic representation of the alignment of the sex factor *cluA-mobA* region with the equivalent regions of conjugative elements identified in *Enterococcus* and *Streptococcus* is shown. The predicted amino acid sequences of the *orfs* shown in colour share 21–88% identity with their homologues found in the other systems and are represented in the same colour. pTEF2-encoded proteins in this region share high-sequence identity (94–100%) with gene products of pCF10 and their genes display identical organization; for these reasons, the pTEF2 genes are not represented in the figure. The genes indicated in black encode proteins that do not share sequence similarity with gene products of the two proven functional DNA transfer systems (the sex factor and pCF10) and do not share sequence similarity with more than two of the other transfer systems. The strains harbouring the chromosomal conjugative element in *L. lactis* and *S. agalactiae* and the two *Enterococcus* plasmids are indicated on the left.

Figure 3: DNA and translated amino acid sequence of *orf18*.

The nucleotide and amino acid sequences correspond to SED ID NOS: 1 and 2, respectively.

Key:
Boxed sequence = transmembrane region
Underlined sequence = highly-conserved domain (HDC)
Double-underlined sequence = cysteine histidine-dependent amidase (CHAP)

Figure 4: Schematic representation of Orf18 and its homologues found in *Enterococcoccus* and *Streptococcus*.

The different domains were predicted by analysing the protein sequences using the SMART [6] search tool and performing a ClustalW alignment [8]. The eight proteins were analysed using various bioinformatics approaches. The cysteine, histidine-dependent amidohydrolase/peptidase or CHAP domain (black rectangle) was predicted by analysing the protein sequences using the InterProScan [41] at http://www.ebi.ac.uk/InterProScan/ with a cut-off *E*-value of 10-5. The transmembrane domain (blue rectangle) was predicted through the TMHMM server v2.0 [42] at http://www.cbs.dtu.dk/services/TMHMM/. The probability for the presence of signal peptides was calculated using SignalP version 3.0 (http://protfun.net/services/SignalP/); no signal peptide was predicted. The eight protein sequences were submitted for multiple sequence alignment using ClustalW2 [43] at the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/clustalw2/) using default settings. The alignment allowed the identification of a highly conserved domain or HCD (red rectangle). The amino acid sequences of HCD are shown in Fig. 12. The scale bar is adjusted to Orf18.

Figure 5: Orf18 peptidoglycan lytic activity.

Orf18 is required for sex factor DNA transfer and leads to cell lysis when overexpressed. **A.** Conjugation frequency measurements were done with different donor strains: the parental strain FI10707 containing the vector pUK200 [wt(vector)], the *orf18*-deleted strain FI10720 containing pUK200 [Δ*orf18*(vector)] and the same strain containing pFI2640 expressing Orf18 under the control of nisin [Δ*orf18*(P*nisA::orf18*)]. Conjugation frequency is expressed as the number of transconjugants per donor cell. Nisin concentration is expressed in ng ml-1.

B. Quantitative RT-PCR: two primers generating a 1.2 kb *orf18* internal fragment were used.

C. SDS-dependent lysis of cells expressing Orf18. The strains used are FI10720 containing the vector pUK200 [Δ*orf18*(vector)], FI10720 containing pFI2640 [Δ*orf18*(P*nisA::orf18*)], the sex factor-deleted strain FI9979 containing the pUK200 vector [Δsex factor(vector)] and FI9979 containing pFI2640 [Δ*sex factor*(P*nisA::orf18*)]. The cells were grown to an OD600 of 0.5 before addition of nisin (ng ml-1) and cells were grown for 16 h. And 0.5% SDS was added to the culture and visible cell lysis could be observed when Orf18 was expressed.

D. Orf18 peptidoglycan lytic activity.

a) AFM image of *Lactococcus lactis* control cells.

b) AFM image of *Lactococcus lactis* cells expressing *orf18*

Figure 6: Release of intracellular *Listeria* endolysin by Orf18 expression

Biological activity of lysin LM4 evaluated by overlaying sample wells with *Listeria monocytogenes*. Clear zones indicate lytic activity against *Listeria*: **1** - buffer control (negative control); **2** - cell extract of *L. lactis* expressing LM4 (positive control); **3** - five fold concentrated culture supernatant of uninduced strain expressing *orf18*; **4** - culture supernatant of nisin induced strain expressing *orf18*; **5** - five fold concentrated culture supernatant of nisin induced strain expressing *orf18*; **6** - five fold concentrated culture supernatant of uninduced strain expressing lysin LM4; **7**- culture supernatant of nisin induced strain expressing lysin LM4; **8** - five fold concentrated culture supernatant of nisin induced strain expressing lysin LM4; **9** - five fold concentrated culture supernatant of uninduced strain carrying *orf18* and lysin LM4; **10** - culture supernatant of nisin induced strain expressing both *orf18* and lysin LM4; **11** - five fold concentrated culture supernatant of nisin induced strain expressing *orf18* and lysin LM4. (Duplicates of 9-11 are *orf18* deletion derivatives lacking the CHAP domain).

Figure 7: *Lactoctoccus lactis* growth and survival following induction of *orf18*

The strain used in this study is FI9979 (sf-, ΔnisA in nisin transposon) harbouring pUK200 as a control or pUK200 containing Orf18 or Orf18 deleted in its CHAP domain. Nisin was added at t = 0h. For the viable counts, different cell dilutions from 2 hours growth were plated on GM17 containing chloramphenicol. The values are the means from triplicate samples (standard deviation, $OD_{600} \leq 9\%$, lysis ≤ 15%).

Figure 8: Transmission electron microscopy of cells expressing Orf18

A. Control cells treated with nisin from a mid-exponential culture (OD600 = 0.4). The two pictures represent different stages of cell division.

B. Left panel: schematic representation of cells treated with ampicillin or expressing Orf18. Right panel: *L. lactis* strain FI10720 containing the pUK200 control vector collected from a mid-exponential culture (OD600 = 0.4) that stopped growing after addition of ampicillin (50 mg ml-1) at an OD600 of 0.2.

C. *Lactococcus lactis* strain FI10720 containing pFI2640 expressing Orf18 collected from a mid-exponential culture (OD600 = 0.4) that stopped growing after addition of nisin 0.5 ng ml-1. The arrows in left pane C show the irregular and thinner newly synthesized cell wall.

D. Orf18 immunodetection in FI10720 cells expressing Orf18. Cells are the same as those shown in (C). A primary rabbit polyclonal antibody against Orf18 was used, followed by a secondary antibody labelled with 5 nm diameter gold particles. No signal was detected for *L. lactis* FI10720 cells containing the control vector. The arrows indicate the black spots corresponding to the detected signals. The samples shown in these pictures are representative examples of all longitudinal sections observed. The different sections were examined and photographed in a JEOL 1200 EX/B transmission electron microscope. Scale bar, approximately 0.5 mm.

Figure 9: Analysis of the relationship between members of the Orf18 (CsiA) protein family

Phylogenetic analysis comparing the relationship between the sex factor Orf18 (CsiA), PrgK and B0020 of *E. faecalis* pCF10 and pTEF2 plasmids, respectively, Gbs1133 and Gbs1359 of *S. agalactiae* NEM316 GIs X and XII, respectively, Sag1286 of *S. agalactiae* Tn*5252*-like, Ssu05–969 and Ssu98–0981 proteins of *S. suis* strains 05ZYH33 and 98HAH33 respectively. The phylogenetic tree was constructed using the neighbour-joining algorithm as a distance-based method.

Figure 10: The HCD domain causes SDS-dependent cell lysis only in growing cells

A. Cells were treated during growth: cells were grown to an OD600 of 0.2 before addition of ampicillin or nisin. After 1.5 h of treatment, the cell cultures reached an OD600 of around 0.4 and cell lysis was assessed by addition of 0.5% SDS. As nisin induction does not affect the growth of the Orf18 (CsiA) R577A mutant, cell lysis was assessed when cells reached an OD600 of 0.4.

B. Cells were treated after growth was stopped: cells were grown to an OD600 of 0.2 and to prevent cells growing further, they were concentrated 40 times before the addition of ampicillin or nisin. Cell lysis was assessed after the period of time indicated by addition of 0.5% SDS on cells diluted with fresh medium to an OD600 of 0.4.

C. Orf18 (CsiA) immunodetection on cell extracts from cells collected 1.5 h after growth was stopped. The arrow on the left indicates the position of the Orf18 (CsiA) protein. The percentage of lysis was defined as follows: OD600 after SDS/OD600 before SDS x 100. The different strains expressing the different variants of Orf18 are *orf18*-deleted strain

FI10720 containing the vector pUK200 (Δorf18), FI10720 containing pFI2640 (Orf18), FI0720 containing pFI2641 (Orf18CHAP) and FI10720 containing pFI2645 (R577A). *P*-values are indicated as follows:

***$P < 0.01$ for a comparison with wt; B. ***$P < 0.01$ for a comparison with ΔCHAP in the same conditions; **$P < 0.05$ for a comparison with ΔCHAP in the same conditions. Amp, ampicillin; wt, wild-type.

Figure 11: Orf18 (CsiA) is a D-Ala–D-Ala carboxypeptidase inhibitor

A. Detection of *L. lactis* Orf18 protein by Bocillin FL. A total of 510 amino acids of the C-terminal region of Orf18 were purified using the His-tag procedure, and different amounts of protein were labelled with the fluorescent penicillin derivative Bocillin FL (25 mM) for 30 min, and loaded on a SDS-PAGE. The protein was visualized using Coomassie blue staining (a) and by using fluorescence imaging (b) (Pharos FX plus, Bio-Rad). The different quantities of protein are indicated.

B. Colorimetric estimation of carboxypeptidase activity in the presence or absence of Orf18. The colorimetric test Penzym (Neogen) based on D-Ala–D-Ala carboxypeptidase inhibition has been used to assess the effect of Orf18 on the enzyme activity. A pink colour indicates the presence of D-Ala as the result of cleavage of the D-Ala–D-Ala peptide bond by the carboxypeptidase. The different assays were performed as follows: a, buffer; b, carboxypeptidase (10 ml) + buffer; c, carboxypeptidase (5 ml) + BSA; d, carboxypeptidase (5 ml) + Orf18 (10 mg); e, carboxypeptidase (10 ml) + Orf18 (10 mg) + BSA (10 mg); f, Orf18 (10 mg) + BSA (10 mg); g, carboxypeptidase (10 ml) + ampicillin (1 mM).

Figure 12: Sequence alignment of the HCD domain of CsiA with its homolog sequences

Identical amino acid residues are in red and their position is indicated with an asterisk, Double dots indicate similar amino acid residues. The two amino acid residues that were subjected to alanine substitution are marked with a rectangle. *Ef, E. faecalis*; *Ll, L. lactis*; *Ss, S. suis*; *Sa, S. agalactiae.*

EXAMPLES

Controlled release of intracellular materials from bacteria by expression of the Lactococcus lactis orf18 *gene*

Summary

This invention is concerned with the properties of the *orf18* gene encoded by a transmissible genetic element (the sex factor) that is integrated within the chromosome of *Lactococcus lactis* MG1363. This species is used extensively for dairy and other food fermentations and the MG1363 strain is used worldwide as a biotechnology platform with diverse applications [1]. The sex factor encodes a gene transfer process that facilitates its own movement between strains of lactococci by conjugation (a highly evolved process dependant on cell to cell contact). We have undertaken a detailed molecular characterisation of the sex factor and the conjugation process that it encodes [2-7]. The invention stems from an unanticipated biotechnological potential of gene *orf18*, which is essential for conjugation.

From its DNA sequence the Orf18 protein is predicted to be membrane associated with a C-terminal peptidoglycan hydrolase domain (cysteine, histidine-dependent aminohydrolase/peptidase, or CHAP, domain). The *orf18* gene was expressed in isolation in *L. lactis* under the control of a heterologous promoter, the P_{nisA} promoter of the nisin biosynthesis operon. This promoter is subject to positive regulation in response to exogenously supplied nisin, with gene expression proportional to the amount of inducer supplied. This provides exquisite "volume control" and tight regulation. When *orf18* was expressed using this system we observed SDS promoted host cell lysis and extensive cell wall damage that was visualised by atomic force microscopy.

This phenotype led to the recognition of several applications of the *orf18* gene, including:

1) Enhanced purification of intracellular products from lactococcal fermentations.
2) GI tract delivery and release of bioactive compounds.
3) Accelerated flavour development in dairy manufacturing processes.
4) Enhanced transformation efficiency.
5) Exploitation of these applications in additional bacterial species.

The *orf18* gene

We have determined the genome sequence of *Lactococcus lactis* MG1363 [8] and confirmed our earlier independent DNA sequence of the 60kb lactococcal sex factor that exits as an integrated genetic element. The conjugation system of the sex factor involves a large cluster of genes. The overall organisation of the lactococcal sex factor and the location of *orf18* are presented in Figure 1. The *orf18* gene is part of a gene cluster that shows some conservation amongst transmissible genetic elements in taxonomically related bacterial species. A BLASTP program run against Orf15, Orf17, Orf18 and Orf20 showed that these four proteins share a high sequence identity (30 to 70%) with gene products of conjugative systems in other Gram positive bacteria: namely, the conjugative plasmids pTEF2 and pCF10 from *Enterococcus faecalis*, the conjugative transposon Tn*5252* of *Streptococcus* and gene products from two uncharacterised regions found in *Streptococcus agalactiae* NEM316 genome. A comparison of these related regions including homologues of *orf18* is shown in Figure 2. The DNA sequence [SEQ ID NO: 1] and the translated amino acid sequence [SEQ ID NO: 2] of *orf18* are shown in Figure 3.

The gene cluster of L. lactis *sex factor encoding the putative DNA transfer apparatus shares homology with five ancestrally related Gram-positive coccal conjugation systems*

Among the 59 predicted genes carried by the *L. lactis* sex factor, we investigated genes encoding proteins involved in the early stages of conjugation, with a particular interest in proteins responsible for the translocation of the sex factor DNA through the cell envelope. Two of the sex factor-encoded proteins have homology with specific components of T4SS. The *orf17* gene (Fig. 2) encodes a putative T4SS VirB4 component, and the *traD* gene encodes the VirD4/TraD T4SS-associated coupling protein. Furthermore, a BlastP2 search against Orf15 encoded by the *orf15* gene revealed that its carboxy-terminal moiety aligns with the putative VirB6 T4SS component of *Pseudomonas syringae* pv. maculicola [44]. These data suggest that this region of the sex factor encodes a T4SS. A comparative analysis of the sex factor with completely sequenced microbial genomes revealed that a group of genes located between and including *cluA* and *mobA* (Fig. 2) shares similarities with gene clusters found in seven other systems, according to their levels of syntheny and gene product homology. These include the two closely related *Enterococcus faecalis* conjugative plasmids pCF10 [35] and pTEF2 [45], copies of the streptococcal conjugative transposon Tn*5252* found in *Streptococcus agalactiae* 2603V/R [46, 47] and in the *S. suis* genomes 05ZYH33

and 98HAH33 [48], as well as two putative conjugative systems that are part of the genomic islands (GIs) X and XII found in the genome of *S. agalactiae* NEM316 [49]. Strong homologies of both DNA and protein sequences between the *prgApcfG* region of plasmid pCF10 and a region of the closely related plasmid pTEF2 were previously reported [50]. For further investigation, we chose to compare each gene product from the six gene clusters, including that of the sex factor against each other. As previously described [71], the *L. lactis* sex factor aggregation protein CluA is similar to the aggregation protein Asc10 of *E. faecalis* [51] encoded by the *prgB* gene of pCF10 (and pTEF2), and to the streptococcal Ssp-5 aggregation factor involved in adhesion to eukaryotic cells [52]. These three proteins share similarities with the gene products of *gbs1143* and *gbs1356* of the two systems found on GIs X and XII of *S. agalactiae* NEM316 respectively (Fig. 2). In *L. lactis*, the *mobA* gene [72] of the sex factor and the *ltrB* gene [53] of the closely related pRS01 conjugative plasmid are coding for a putative relaxase enzyme. Both *mobA* and *ltrB* genes are interrupted by the identical LI.ltrB group II intron maturase gene (*matR*) likely to represent a single insertion event in a common ancestor strain [54]. This feature is unique to *L. lactis* as the predicted relaxase genes in the five other conjugative systems are not found to be disrupted. In addition to CluA and MobA, the sex factor-encoded Orf15, Orf17, Orf18, Orf20, Orf24, TraD, Orf28 and Orf34 protein sequences share identity with gene products found in the five other systems (Fig. 2). Interestingly, the *orf28* and *ltrC* gene products of the sex factor are similar to the amino- and carboxy-terminal region, respectively, of a unique predicted polypeptide chain encoded by the three following systems: the *pcfD*, *orf26* and *gbs1126* of pCF10, pTEF2 and island X of *S. agalactiae* respectively. The amino-terminus of these proteins aligns with bacterial primases (COG0358) and their carboxy-terminal region with anti-restriction enzymes (COG4227). As the two interrupting genes *orf29* and *orf30* are found only in *L. Lactis* (Fig. 2), we propose that an insertion event of these two genes occurred within a single ancestor gene leading to the formation of the two separate ORFs, *orf28* and *ltrC*. We compared the gene organization of the six systems (Fig. 2) and defined two groups. The first group shows a gene organization similar to the one found between the *cluA* and *mobA* genes of the sex factor and includes the *Enterococcus* plasmids pCF10 and pTEF2, as well as the GI X of *S. agalactiae*. The second group with a different conserved gene organization is composed of the conjugative systems of GI XII and the copy of Tn*5252*. In contrast to group 1, the *traD* orthologues in group 2 are located upstream of those of *orf17* and *orf18*, whereas the *cluA* orthologues are located downstream. To investigate the relationship between the different conjugative systems further, we chose to perform a phylogenetic study of the

conserved gene product Orf18 and its orthologues found in ancestrally related systems, including the two Tn*5252*-like copies found in *S. suis*. As predicted from the gene organization of their respective conjugative systems, this study confirmed the assignment of Orf18, PrgK, B0020 and Gbs1133 to the same group (Fig. 9). Although the second group still includes all orthologues found in Tn*5252*-like copies, Gbs1359, whose conjugal system (GI XII of *S. agalactiae*) was previously classified in this group on the basis of gene syntheny, appears to constitute a third group indicating some evolutionary divergence between the Tn*5252*-like DNA transfer apparatus of *S. agalactiae* 2603V/R and *S. suis* and their counterpart in *S. agalactiae* NEM316. Interestingly, our results revealed a close relationship between a group of genes of the sex factor and elements found in Gram-positive cocci; the human pathogens *E. faecalis* and *S. agalactiae*, and the pig pathogen *S. suis*. In addition, the conjugative plasmid pCF10 has been shown to be involved in *E. faecalis* virulence [35, 36] and the streptococcal GIs X and XII, which harbour virulence genes, have been proposed to constitute pathogenicity islands [37]. While those ancestrally related DNA molecules have acquired virulence traits that contribute to the fitness of their bacterial host, the lactococcal sex factor has not yet been proven to confer any advantage to its host *L. lactis* and seems to have evolved as a selfish DNA molecule [55].

Orf18 is a large protein (870aa) containing an amino terminal membrane-spanning domain. A SMART search [9] identified functional domains in Orf18 and its five homologues. All these proteins have a similar architecture with the membrane-spanning domain at the same location within the amino terminal region indicating that they are likely to be membrane-anchored. They all contain an amidase domain (CHAP: cysteine, histidine-dependent aminohydrolase/peptidase domain) described as a peptidoglycan hydrolase [10] at their carboxy terminus and none of them has a typical signal peptide. Protein sequence alignment of all six homologues allowed us to define a distinct highly conserved domain (HCD) of 95 amino acids (previously thought to be 130 amino acids) that shares no homology with any other proteins from the protein databases. These features are summarized in Figure 4.

Phenotypes associated with *orf18*

To investigate the role of *orf18* in conjugal transfer, an in-frame unmarked deletion of the gene was constructed in a parental strain containing a tetracycline resistance (*tet*) selection marker gene integrated in a non-essential region of the lactococcal sex factor.

DNA transfer was completely impaired in the *orf18* mutant (see Table 4 below). For complementation studies, the *orf18* gene was cloned on a plasmid and expressed under the control of the nisin inducible P_{nisA} promoter in the *orf18* deletion mutant. Complementation of the *orf18* gene in this strain restored conjugal activity confirming that only *orf18* had been affected in the deletion mutant. No addition of nisin was required to restore conjugation (see Table 4 below) showing that the very low P_{nisA} promoter basal activity supplied sufficient Orf18 to restore the phenotype. This indicates that conjugal activity is based on finely tuned protein levels. Quantitative RT-PCR experiments showed that *orf18* was transcribed in the absence of nisin induction. Interestingly, induction of *orf18* by the addition of nisin resulted in significantly lower levels of DNA transfer in this strain.

We observed that this decrease in DNA transfer reflects the toxic effect of Orf18 overexpression. Only 10% of the donor cells remained viable after a 10 $ng.ml^{-1}$ nisin induction, which explains the lower conjugation frequency. The presence of the CHAP domain at the C terminus of the protein suggested that Orf18 might play a role in cell wall degradation, perhaps associated with the movement of transferred DNA between donor and recipient cells during conjugation. Addition of detergent to *orf18*-expressing cells caused their lysis and this phenomenon involved no other sex factor component as *orf18* expression in a sex factor negative background also led to cell lysis after detergent treatment (Figure 5C, see Table 4 below). Atomic Force Microscopy (AFM) was used to show that the cell wall integrity was no longer maintained in the sex factor negative strain overexpressing the *orf18* gene and this is likely to be a consequence of the peptidoglycan degradation caused by Orf18 (Figure 5D).

We investigated the role of different domains in Orf18. Two Orf18 derivatives with C-terminal deletions were constructed and the impact on conjugation frequency and cell lysis was investigated. Expression of Orf18 truncated in its C-terminal CHAP domain showed that sex factor transfer was dramatically reduced but cell lysis was comparable to that of the full length Orf18 protein (see Table 4 below). This indicates that the CHAP domain is not necessary to induce peptidoglycan lysis in the donor strain. Orf18 derivatives truncated for both CHAP and HCD domains were impaired in both DNA transfer capacity and donor cell lysis ability (see Table 4 below) and thus HCD plays a role in donor cell wall degradation. We confirmed this by constructing an Orf18 mutated protein in which the conserved residue arginine 577 in the HCD domain was substituted

by an alanine residue. The protein lost its capacity to cause cell lysis and the DNA transfer efficiency was significantly reduced (see Table 4 below).

Release of an intracellular protein by Orf18

In order to demonstrate the potential of *orf18* to facilitate the release of intracellular proteins from *L. lactis* we have undertaken a proof of concept experiment. For this we used a *Listeria* bacteriophage endolysin that has potential in its own right as a biological control agent [11]. We constructed a series of strains in which the *Listeria* endolysin gene was integrated into the chromosome of *L. lactis* under the control of the lactose promoter [12] and *orf18* was present on a plasmid under the control of the inducible nisin promoter. With this arrangement, *orf18* would be switched off but it could be induced by the addition of nisin. The effect of Orf18 on the release of biologically active *Listeria* endolysin was investigated and the data are presented in Figure 6. The results also demonstrate that this release process does not depend on the CHAP domain and the phenotype involves a more subtle process than just the provision of a cell wall lytic enzyme encoded by *orf18*.

Exemplary uses of the host cells of the invention

1) Purification of intracellular products

The ability of Orf18 to release intracellular material by controlled lysis without the need for the use of an external lytic agent can be exploited in the purification of intracellular products of fermentation where both the extent of lysis and its timing could be subject to precise control.

2) GI tract delivery vehicle for bioactive compounds

Lactococcus lactis has significant potential as a GI tract delivery vehicle for a variety of bioactive compounds, including vaccine antigens, immune modulators and antimicrobials. This potential is very well established by Lothar Steidler and colleagues who have demonstrated remission in ulcerative colitis following the oral administration of *L. lactis* MG1363 derivatives that express the anti-inflammatory cytokine interleukin 10. Importantly this has been achieved both in an animal model [13] and in human trials [14, 15]. In this work bioactive delivery was sub-optimal and we believe *orf18* offers a unique solution. For GI tract delivery the mode of bioactive release may be critical. Intracellular expression might prevent effective delivery and *secA*-dependent secretion is likely to be inefficient within the GI tract. The use of *orf18* to facilitate timed cell leakage makes it possible to deliver an intracellular "payload" of a bioactive compound to the GI tract.

3) Accelerated flavour development for matured cheeses

The present invention also has application in accelerated flavour development for matured cheeses in the dairy industry. Previously we developed an acceleration concept involving induced intracellular enzyme release [16]. When milk is fermented to make cheese, robust lactic acid bacteria are used as starter cultures in a primary fermentation that converts lactose (milk sugar) into lactic acid. Subsequently, during maturation, the slow development of cheese flavour depends on the pool of intracellular enzymes present within the starter bacteria. Maturation is dependent on intracellular enzyme release from the intact starter cells and it is rate limiting. Orf18-induced lysis of starter lactic acid bacteria after primary fermentation but early in the maturation process is an attractive acceleration strategy.

4) Enhanced DNA transformation

Osmotic buffering of cells during Orf18 expression would result in cell wall degradation without cell lysis. It is anticipated that this would ultimately result in protoplast formation and this process, perhaps in moderation and associated with electroporation, could facilitate more efficient DNA transformation in a microbe that is the subject of intense biotechnological interest.

Experimental details

Media, growth conditions and transformations

E. coli was grown at 37°C in Luria-Bertani medium [17] supplemented with 15 μg/ml of chloramphenicol. *L. lactis* strains were grown at 30°C in M17 medium [18] with 0.5% glucose (GM17) or 0.5% sucrose (SM17). Antibiotic-resistance markers in *L. lactis* were selected using chloramphenicol 5 μg ml^{-1}, tetracycline 5 μg ml^{-1}, streptomycin 200 μg ml^{-1} or rifampicin 200 μg ml^{-1}. *E. coli* and *L. lactis* electrocompetent cells were prepared and transformed by the methods of Dower *et al.* [19] and Holo and Nes [20], respectively.

Bacterial strains and plasmids

Escherichia coli strain TG1 [21] was used for cloning experiments and plasmid propagation. All *L. lactis* strains and all plasmids used in this study are described in Tables 1 and 2.

Table 1

Lactococcus lactis strains and plasmids used in the analysis of *orf18*

Strain or plasmids	Relevant characteristics*	Source or reference
Strains		
FI8164	MG1363 with tetracycline marked sex factor, Tet^r	Gasson *et al.* (1995) [3]
FI9979	Transconjugant in FI9012 background, sf neg, *nisA*$^-$, *suc*$^+$, Str^r Rif^r	Stentz *et al.* (2004) [4]
FI10703	FI9979pUK200, Cm^r, sf neg, , *nisA*$^-$, *suc*$^+$, Str^r, Rif^r	This Example
FI10704	FI9979pFIorf18, Cm^r, sf neg, , *nisA*$^-$, *suc*$^+$, StrR RifR	This Example
UKLc10	*Lactococcus lactis* IM16, *pepN*::*nisRK*	Wegmann *et al.* (1999) [27]
FI10706	UKLc10 spontaneous resistant to Streptomycin	This Example

FI10707	UKLc10 Str^r containing a Tet-marked sex factor	This Example
FI10720	FI10707 containing an in-frame deletion in *orf18*	This Example
Plasmids		
pOri280	Eryr, RepA-*ori* + of pWV01, replicates only in strains with *repA in trans*	Leenhouts *et al.* (1996) [26]
pVE6007	Cmr, RepA.; ts, derivative of pWVO1	Maguin *et al.* (1992) [32]
pFI2648	pOri280 containing *csiA* deletion	Stentz *et al.*, 2009 [34]
pUK200	Cmr, P*nisA*, pSH71 replicon	Wegmann *et al.* (1999) [27]
pFI2640	Cm^r, pUK200, *PnisA*::*orf18*	This Example
pFI2641	Cm^r, pUK200, *PnisA*::*orf18* ΔCHAP	This Example
pFI2642	Cm^r, pUK200, *PnisA*::*orf18* ΔHCD + CHAP	This Example
pFI2644	Cmr, pUK200, P*nisA*::*csiA* K576A	Stentz *et al.*, 2009 [34]
pFI2645	Cm^r, pUK200, *PnisA*::*orf18* R576A	This Example
pFI2646	Cmr, pUK200, P*nisA*::*csiA* K576A, R577A	Stentz *et al.*, 2009 [34]
pFI2650	Amp, pET-15b containing the 3′ end of *csiA* fused to the 5′ His-tag sequence	Stentz *et al.*, 2009 [34]

* Tet, tetracycline; Cm, chloramphenicol; Ery, erythromycin; Str, streptomycin; Rif, rifampicin; sf neg, sex factor negative; *nisA*$^-$, *nisA* negative ; *suc*$^+$, sucrose positive.

Table 2

Lactococcus lactis strains and plasmids used in the demonstration of *Listeria monocytogenes* endolysin release by *orf18*

Strain or plasmids	Relevant characteristics*	Source or reference
Strains		
FI7800	Lac+, EmR, MG5267 carrying EmR and LM-4 lysin *genes* in *lacG*	Payne *et al.* (1996) [12]
FI9979	Transconjugant in FI9012 background, sf neg, *nisA*$^-$, *suc*$^+$, StrR RifR	Stentz *et al.* (2004) [4]
FI10703	FI9979pUK200, CamR, sf neg, *nisA*$^-$, *suc*$^+$, StrR RifR	This Example
FI10704	FI9979pFI2640, CamR, sf neg, *nisA*$^-$, *suc*$^+$, StrR RifR	This Example
FI10705	FI9979pFI2641, CamR, sf neg, *nisA*$^-$, *suc*$^+$, StrR RifR	This Example
FI10717	Lac+, EmR, FI10703 derivative containing the lactose operon of FI7800 carrying EmR and LM-4 lysin *genes* in *lacG*	This Example
FI10718	Lac+, EmR, FI10704 derivative containing the lactose operon of FI7800 carrying EmR and LM-4 lysin *genes* in *lacG*	This Example
FI10719	Lac+, EmR, FI10705 derivative containing the lactose operon of FI7800 carrying EmR and LM-4 lysin *genes* in *lacG*	This Example
Plasmids		
pUK200	CamR, *PnisA*, pSH71 replicon	Wegmann *et al.* (1999) [27]
pFI2640	pUK200, *PnisA*::*orf18*	This Example
pFI2641	pUK200, *PnisA*::*orf18ΔCHAP*	This Example

* Ery, erythromycin; Cam, chloramphenicol; Str, streptomycin; Rif, rifampicin; sf neg, sex factor negative; *nisA*$^-$, *nisA* negative ; *suc*$^+$, sucrose positive

The FI10720 strain is an *orf18*-deleted donor strain containing a marked sex factor and was obtained as follows. First, a spontaneous streptomycin resistant derivative of strain UKLc10 (containing the *nisRK* two-component regulatory system [26]) was selected on

GM17 agar plates containing streptomycin giving strain FI10706. FI10706 was used as a recipient in a conjugation experiment where strain FI8164 containing a tetracycline resistance marker integrated into the sex factor was used as a donor. The conjugation experiment leading to sex factor transfer was performed as described below. The transconjugant FI10707 was selected on GM17 containing both tetracycline and streptomycin and used as a background strain to generate the *orf18* deletion.

Construction of an orf18 in-frame deletion

Two in-frame deletions were generated by a recombinant PCR technique [22]. Three individual PCR amplifications were used to create a deletion. The sequences of the oligonucleotide primers used to create the *orf18* deletion are given in Table 3.

Table 3

Oligonucleotide primers

Primer name	Sequence
Orf18dc1[b]	5'-GTCAGGATCCAGCTCTAGGAGGTATCATTC-3' [SEQ ID NO:3]
Orf18dc2	5'-TCATTCCTGCCTGGACATTGAGCGAAGCTT-3' [SEQ ID NO:4]
Orf18dc3	5'-CAATGTCCAGGCAGGAATGACATTTGTTCA-3' [SEQ ID NO:5]
Orf18dc4[c]	5'-GTCACCATGGGGTTCTACTAATGTTATCGC-3' [SEQ ID NO:6]
Orf18-am[c]	5'-GCATCCATGGATAAAAGAAGCAAAGA-3' [SEQ ID NO:7]
Orf18-av[b]	5'-GCATGGATCCCAGTTATTTCTTCCATTTC-3' [SEQ ID NO:8]
Orf18ChapDel[b]	5'-GCATGGATCCATAGCCATTACCAGGCCATC-3' [SEQ ID NO:9]
Orf18HCDDel[b]	5'-GCATGGATCCATAGGTCATATTAAGACTTG-3' [SEQ ID NO:10]
HC-Rala	5'-AATCAATCCTAAGGCAGCTGAAGGAGATTA-3' [SEQ ID NO:11]
HC-Ralarev	5'-TAATCTCCTTCAGCTGCCTTAGGATTGATT -3' [SEQ ID NO:12]

a. Restriction enzyme cleavage sites are underlined
b. Primer tailed with restriction site *Bam*HI
c. Primer tailed with restriction site *Nco*I

First, the orf18dc1 oligonucleotide with a priming site located 800 bp upstream of the gene of interest was paired with orf18dc2 oligonucleotide located at the 5'-end of this gene. This mutagenic primer contained an in-frame fusion of the 5'-end of the gene with its 3'-end. A separate PCR was set up using the orf18dc3 oligonucleotide complementary to the mutagenic primer paired with the orf18dc4 oligonucleotide with a priming site located 800 bp downstream of the gene. The two resulting PCR fragments

were mixed together and used as the template for a third reaction in which orf18dc1 and orf18dc4 outer primers were used to create a "long" PCR fragment spanning the deletion. The resulting PCR fragment was cloned into the integrative plasmid pOri280, leading to pFI2648. The latter plasmid was used in a gene inactivation protocol previously described by Leenhoots *et al.* [24]. An in-frame deletion was made in the *orf18* gene of FI10707 containing the pVe6007 helper plasmid giving the mutant strain FI10720.

Plasmid constructs

The expression vector pUK200 was used for cloning experiments in *E. coli* TG1 [21] or in *L. lactis* FI10720. All the pUK200-based plasmids carrying *orf18*, the deleted variants of *orf18* and the resulting plasmids are listed in Table 1. The different inserts were obtained by PCR using the oligonucleotide primers listed in Table 3. The PCR products were then digested with *Nco*I and *Bam*HI and inserted into the *Nco*I/*Bam*HI restriction endonuclease cleavage sites of pUK200 plasmid giving the three plasmids pFI2640-42. The pFI2645 plasmid was obtained by cloning a PCR fragment resulting from a recombinant PCR procedure (described in the preceding paragraph) into the *Nco*I/*Bam*HI endonuclease cleavage sites of pUK200. The combination of the complementary mutagenic primers HC-Rala/HC-Ralarev with the outer primers Orf18-am and Orf18-av (Table 3) were used to generate the mutated *orf18* variant.

Sex-factor transfer

The different donor strains were grown overnight and diluted 100 times in GM17 containing chloramphenicol 5 $\mu g\ ml^{-1}$. When required, nisin (Aplin and Barret, Trowbridge, UK) was added to the culture medium when the cells reached an OD_{600} of 0.5. The recipient strain FI9979 was grown under the same conditions but in the absence of nisin. Cell mating was triggered by centrifugation of a mixture of donor and recipient strain grown to an optical density at 600 nm (OD_{600}) of 0.8 as previously described by Stentz *et al.* [4]. Donor and recipient cells were mixed at a 1:10 ratio, respectively, and the mixture was centrifuged at 5000 rpm for 5 minutes. The pellet was resuspended in the initial volume of M17 and left for 1 hour at 30°C. Cells were then diluted and plated on selective media containing rifampicin, tetracycline or both antibiotics.

Bocillin FL binding assay and carboxypeptidase colorimetric assay

The binding of the fluorescent penicillin derivative Bocillin FL (Invitrogen) to Orf18 was assessed as described by Zhao *et al.* (1999) [39]. Briefly, 25 mM of Bocillin FL was mixed with different amounts of the purified C-terminal region of Orf18. The reaction mixture was incubated for 30 min at 35°C and boiled for 3 min after addition of SDS-PAGE sample buffer. The different samples were subjected to SDS-PAGE, and the protein was visualized by using fluorescence imaging (Pharos FX plus, Bio-Rad). To assess the capacity of Orf18 to prevent the action of PBPs, the colorimetric DD carboxypeptidase inhibition test (Penzym, Neogen) for detecting β-lactam antibiotics in milk was adapted to this study. The principle of the method previously described by Frère *et al.* (1980) [40] relies on the ability of the D-Ala–D-Ala carboxypeptidase produced by the bacterium *Actinomadura* R39 to cleave the N-terminal D-Ala–D-Ala peptide bond of a peptide substrate. In this assay, the intensity of the pink colour obtained after incubation depends on the concentration of D-Ala produced in the reaction mixture resulting from the D-Ala–D-Ala peptide bond cleavage by the carboxypeptidase. A substrate tablet containing the peptide substrate and the reagents was re-suspended in 1 ml of water and vortexed for 1 min. The suspension was centrifuged and the supernatant was used for the assay. Ninety microlitres of buffer (Tris-HCl 50 mM, EDTA 1 mM, KCl 100 mM, pH 7.3) or 90 ml of buffer containing 10 mg of BSA or/and 10 mg Orf18 was added to 10 ml of carboxypeptidase. Samples containing only buffer or the carboxypeptidase in the presence of 1 mM of ampicillin were used as negative controls. A sample containing Orf18 in the absence of carboxypeptidase was also included. One hundred microlitres of substrate solution was added and the mixture incubated for 20 min at 47°C. Subsequently, differences in colour between the different samples were assessed visually.

Results

The Orf18 Phenotype

Strain FI10707 carries a sex factor marked with a tetracycline resistance gene and in the derivative strain FI10720 *orf18* has been deleted. As shown in Table 4, deletion of *orf18* completely impairs conjugation but this can be restored by the introduction of a plasmid expressing *orf18* under the control of the nisin inducible promoter P_{nisA} [26]. Unexpectedly, complementation of the conjugation phenotype is poor when the nisin

promoter is induced. This reflects the impact of orf18 on the cell surface and the associated lysis that occurs. Constructs in which the sex factor was absent from the chromosome were used to prove that this cell wall degradation and lysis phenotype is associated with orf18. As shown in Figure 5C, lysis upon addition of membrane disruptive SDS was readily demonstrated when orf18 was expressed. Damage to the cell wall was visualized by Atomic Force Microscopy and this is illustrated in Figure 5D.

Table 4

Conjugation and cell lysis phenotypes associated with the expression of Orf18 and Orf18 variants

Strain Number	Genotype	Tet^R transfer frequency	Inducible cell lysis
FI8164	Wild type sex factor	10^{-2}	-
FI10708	*orf18* deletion	0.4×10^{-7}	-
FI10709	*orf18* deletion complemented by nisin controlled *orf18*	6.7×10^{-3}	+
FI10710	*orf18* deletion complemented by nisin controlled *orf18* with CHAP domain deleted	3.5×10^{-5}	+
FI10711	*orf18* deletion complemented by nisin controlled *orf18* with CHAP & HCD domains deleted	2.0×10^{-7}	-
FI10714	*orf18* deletion complemented by nisin controlled *orf18* with R577 A mutation	1.4×10^{-4}	-

In order to more precisely identify the functional domain associated with this phenotype, two carboxy terminal deletions were made. One deleted the CHAP domain (in plasmid pFI2641) and the second deleted both the CHAP and HCD domains (in plasmid pFI2642) as defined in Figure 4. The cell wall lytic phenomenon was retained in the absence of the CHAP domain whereas it was eliminated in the case of the larger deletion carried by pFI2642. The active region of orf18 was defined further by the construction of a site directed mutation (R577A) within the HCD domain (in plasmid pFI2645). This single amino acid change eliminated the cell wall lysis phenotype. Table 4 summarizes the properties of these various *orf18* constructs.

We have undertaken a more detailed analysis of the impact of orf18 expression on cell growth, cell viability and the release of an intracellular marker enzyme. This involved

subjecting a growing lactococcal culture to different levels of nisin induction and subsequently monitoring changes to optical density and viable count. As shown in Figure 7 the results indicate that controlled *orf18* expression can be used to create a cell lysis and intracellular release phenotype. We have undertaken an analysis of the impact of orf18 expression on the release of the intracellular marker enzyme lactate dehydrogenase. This involved subjecting a growing lactococcal culture (optical density 0.15) to different levels of nisin induction and subsequently monitoring the release of LDH into the culture medium. As shown in Table 5, controlled *orf18* expression can be used to create a "cell leakage" phenotype. It is remarkable that cells expressing Orf18 at levels that do not affect growth (0.5 ng/ml nisin) do release 5.8 times more LDH than the control cells. Higher levels of Orf18 expression that affect growth but not survival (1 ng/ml nisin) allow 7.8 times the LDH levels released by the control cells, demonstrating growth conditions of Orf18-expressing cells providing efficient intracellular protein release without affecting cell viability.

Sytox Green DNA detection dye was used to quantify the release of chromosomal DNA into the medium as a consequence of cell lysis using flow cytometry. The experiment was performed with the conditions indicated below (Table 5). Nisin concentrations of 0.5 ng/ml and 2.5 ng/ml were added into the culture medium of FI10703 and FI10704. No significant difference in DNA release was measured between FI10703 and FI10704 incubated with 0.5 ng/ml nisin. This result indicates that the 5.8-fold LDH release measured for FI10704 in these conditions is the consequence of cell leakage rather than cell lysis that could also explain why the cell growth is not affected. On the other hand, a significant increase in the amount of DNA released from FI10704 grown in the presence of nisin 2.5 ng/ml was measured. In this case, the 5.2-fold-increase of LDH release measured in the supernatant is likely to result from a combination of cell lysis and cell leakage.

No difference in terms of DNA release was observed before and after centrifugation indicating that although cell structure was made fragile following the action of Orf18, the cell integrity was maintained and centrifugation does not affect the results of intracellular protein release.

Table 5

Evaluation of LDH release

Strain	FI10703	FI10704			
Nisin (ng/ml)	10	0.5	1	2.5	10
OD_{600nm}	0.85	0.88	0.59	0.38	0.15
LDH (IU/L)	2.1	12.1	16.5	11.0	2.0

The strains used in this study are FI10703 (sf-, ΔnisA in nisin transposon harbouring pUK200) as a control and FI10704 (sf-, ΔnisA in nisin transposon harbouring pFI2640). Nisin was added when cells reached an OD_{600} of 0.15 and the cells were incubated for 2 hours at 30°C. 10 ml of culture was then centrifuged at 5000 rpm for 10 minutes and the supernatant was collected. A colorimetric LDH assay (Bioassay Systems, Hayward, USA) was used to assess the release of the enzyme into the culture supernatant. The values are the means from triplicate samples (standard deviation, $OD_{600} \leq 11\%$, IU/L LDH $\leq 7\%$).

Orf18 expression leads to cell wall synthesis inhibition and prevents cell division

We observed that cells in which Orf18 expression was induced with nisin at an OD_{600} of 0.2 stopped dividing when they reached an OD_{600} of 0.4, i.e. after one generation. Addition of SDS to the culture led to visible cell lysis. The same phenotypes were observed when ampicillin (50µg/ml), a cell wall synthesis inhibitor, was added to control cell cultures (that do not express Orf18) at an OD_{600} of 0.2. These results suggested strongly that like ampicillin, Orf18 could interfere with the synthesis of the cell wall, thus preventing further cell division. Transmission electron microscopy observations of cells that stopped dividing after ampicillin treatment or Orf18 expression showed clearly that newly synthesised cell wall and septum formation were impaired in both cases (Figure 8). Furthermore, the fractions of cell wall that were newly synthesised in the presence of ampicillin or Orf18 were unable to maintain the local cell shape leading to the formation of bulges. This result confirmed that Orf18 is involved in cell wall synthesis inhibition.

Orf18 is required for sex factor DNA transfer and exhibits detergent-mediated lytic activity when overexpressed

To investigate the role of Orf18 in conjugal transfer, an in-frame unmarked deletion of the *orf18* gene was constructed in a parental strain containing a tetracycline selection marker gene integrated in a nonessential region of the sex factor [3]. DNA transfer was completely impaired in the *orf18* mutant FI10720 (Fig. 5A). Complementation of the *orf18* deletion restored conjugal activity, confirming that only the deletion of *orf18* is responsible for the loss of conjugation ability. We would like to stress that no addition of nisin was required in order to restore conjugation, showing that the P*nisA* promoter's basal activity described in other works [56] resulted in a sufficient level of Orf18 to restore the phenotype. This was confirmed by quantitative RT-PCR (Fig. 5B) and suggests that conjugal activity is based on finely tuned Orf18 levels. Interestingly, addition of nisin significantly decreased the donor cells culturability (data not shown), resulting in lower conjugation frequencies. For instance, only 10% of the donor cells were still culturable after induction of *orf18* expression by the addition of 0.1 ng ml-1 nisin, leading to a 10-fold decrease in conjugation (Fig. 5A). We hypothesized that the presence of the CHAP domain and its potential PG hydrolase activity would lead to cell wall damage when Orf18 is overexpressed, and cell wall damage could then be monitored by measuring cell lysis. A common procedure for causing bacterial cell lysis

uses the denaturing detergent sodium dodecyl sulphate (SDS). To achieve SDS cell lysis in *L. lactis*, cells normally require pretreatment with lysozyme [57] resulting in the weakening of the bacterial cell wall and causing the cell to burst subsequently. However, the action of lysozyme is not necessary in *orf18*-expressing cells, as the addition of SDS caused immediate visible cell lysis (Fig. 5C). Cells expressing Orf18 do not require an enzymatic digestion of their cell wall to allow SDS-lysis, suggesting, that like lysozyme, Orf18 promotes cell wall weakening. Orf18 was expressed in a strain lacking the sex factor and the potential of the protein to cause cell lysis was assessed. Figure 5C indicates that Orf18 is the only sex factor component required for cell lysis. At this stage of the study, we hypothesized that the CHAP domain of Orf18 was responsible for the loss of cell culturability and for SDS-dependent cell lysis. We pursued our Orf18 investigation by measuring the impact of a CHAP domain deletion on sex factor transfer and cell lysis.

The highly conserved domain of Orf18 is responsible for the donor cell lysis phenotype

The presence of a CHAP domain at the C-terminus of Orf18 suggested a possible role of this domain in SDS-dependent cell lysis. Three different carboxy-terminal deletions of Orf18 were constructed and their impact on both cell lysis and conjugation frequency was measured (Table 6). Expression of Orf18 truncated in its carboxy-terminal CHAP domain (on pFI2641) showed that sex factor transfer was attenuated 180-fold compared with the parental strain, but cell lysis was comparable to that observed with the full-length Orf18 protein. This was a surprising result as it implies that the CHAP domain is not necessary to cause cell lysis. However, the activity of this domain is necessary to facilitate DNA transfer. Orf18 derivatives truncated for both HCD and CHAP domain (on pFI2642) were impaired in their DNA transfer capacity leading to a 3 x 10^4-fold reduction in transfer compared with the parental strain. Furthermore, no cell lysis of the donor could be observed, suggesting that the HCD plays a role in donor cell wall weakening. Diverse *in vitro* approaches (such as zymogram electrophoresis and bacterial lysis assays) failed to demonstrate the ability of the purified Orf18 to degrade PG (data not shown). It is possible that due to its size, the protein needs to be properly incorporated into the membrane to allow efficient cell wall degradation. Alternatively, Orf18 might act locally by activating a PG hydrolase in its vicinity or by preventing PG synthesis. The HCD of Orf18 seems to play an important role in conjugation and cell lysis. To confirm this, we constructed point mutations within this domain. The alignment of the HCD from Orf18 with HCDs from seven of Orf18's orthologues enabled us to select conserved

amino acid residues for site-directed mutagenesis (Fig. 12). We chose to replace the two neighbouring positively charged amino acid residues lysine and arginine (K and R) (Fig. 12). We postulated that these two positively charged residues could play a role in the interaction of Orf18 with the negatively charged cell wall. We constructed three mutated proteins: a first variant with the lysine 576 replaced by an alanine, a second variant with the arginine 577 replaced by an alanine and a third variant, where both residues were replaced by alanine residues. The DNA transfer and cell lysis abilities of the mutated proteins were assessed. No effect on either cell lysis or sex factor transfer could be observed for the Orf18 K576A mutant protein (Table 7), whereas no cell lysis was observed in Orf18 R577A and the sex factor transfer frequency was reduced 45 times compared with the wild-type protein. The same phenotypes were observed for the double-mutant confirming that the arginine 577 mutation was responsible for the effect on Orf18. These results show that the HCD is linked to high frequency conjugation.

Table 6

Mapping Orf18 functional domains by generating carboxy-terminal deletions

Plasmid[a]	Orf18 carboxy-terminal deletion[d]	Cell lysis[e]	Sex factor transfer[f]
pFI2640[b]		+	$6.3 \times 10^{-3} \pm 0.3$
pFI2641		+	$3.5 \times 10^{-5} \pm 0.6$
pFI2642		-	$2 \times 10^{-7} \pm 0.9$
pUK200[c]		-	$< 0.4 \times 10^{-7}$

a. Plasmids expressing the deleted variants of Orf18 in the *orf18*-deleted mutant strain FI10720.

b. Plasmid expressing the wild-typeOrf18.
c. Control empty vector pUK200.
d. Schematic representation of Orf18 with its different carboxy-terminal-deleted variants.
e. Cell lysis was assessed by measuring the OD600 of the culture before and after addition of 0.5% SDS.
The symbol '+' indicates the observation of visible cell lysis after addition of SDS with a 40% decrease in OD600.
The symbol '-' indicates that no visible cell lysis could be observed after addition of SDS with a decrease in OD600 < 4%.
f. Sex factor transfer was performed as described in *Experimental procedures.* The values (transconjugants/donor) represent the average of three independent assays.

Table 7

Sex factor DNA transfer and cell lysis following expression of the *orf18* variants

orf18 allele[a]	Cell lysis[b]	Sex factor transfer[c]
Wild-type	+	67 ± 9
K576A	+	64 ± 4
R577A	-	1.4 ± 0.9
K576A, R577A	-	1.7 ± 0.7

a. The different alleles of *orf18* carried by an expression vector in the *L. lactis* donor strains are indicated.

b. Cell lysis was assessed by measuring the OD600 of the culture before and after addition of 0.5% SDS. The symbol '+' indicates the observation of visible cell lysis after addition of SDS with a 40% decrease in OD600. The symbol '-' indicates that no visible cell lysis could be observed after addition of SDS with a decrease in OD600 < 4%.

c. Sex factor transfer was performed as described in *Experimental procedures*. The values ($x10^{-4}$ transconjugants/donor) represent the average of three independent assays.

Orf18 expression leads to cell lysis solely on growing cells

The HCD of Orf18 plays an essential role in the detergent-mediated cell lysis. Whether Orf18-promoted cell lysis was cell growth-dependent needed to be determined. As the β-lactam antibiotic ampicillin is an inhibitor of PG synthesis that requires cell growth to cause *E. coli* cell lysis [58], we included bacterial cells treated with ampicillin as a control. As expected, cells treated with ampicillin were susceptible to lysis only in the case of growing bacteria (Fig. 10A and B). Derivatives of the Δ*orf18* strain FI10720 expressing *in trans* either the wild-type Orf18, Orf18 deleted in its CHAP domain or Orf18 containing the R577A mutation in its HCD were tested for their ability to promote cell lysis under both growing and non-growing conditions. Expression of the different Orf18 variants was induced by the addition of nisin when cells reached an OD600 of 0.2. Growing cells expressing Orf18, Orf18 deleted in its CHAP domain and cells treated with ampicillin all stopped growing when they reached an OD600 of approximately 0.4, i.e. after having completed one further generation. Addition of SDS to those cells resulted in significant cell lysis (Fig. 10A). As expected, cells expressing Orf18 R577A were neither affected in their growth nor did they lyse. Orf18 expression was confirmed by immunodetection of the protein in cell extracts obtained from strains expressing the entire protein (Fig. 10C). Unlike growing cells induced for 1.5 h, addition of SDS to non-growing cells treated with

ampicillin or induced with nisin for the same period of time did not lead to significant cell lysis (Fig. 10B). Interestingly, longer periods of incubation (3 and 16 h) led to significant cell lysis exclusively in non-growing cells expressing Orf18 still containing the CHAP domain (wild-type Orf18 and Orf18 R577A). This appears to be the result of PG lytic activity from the CHAP domain as the HCD has been found to act only on growing cells.

Orf18 is a D-Ala–D-Ala carboxypeptidase inhibitor

Our results show that Orf18 is located in the PG and strongly suggest that it does inhibit PG biosynthesis. These observations led us to consider the possibility that Orf18 could directly inhibit the activity of penicillin binding proteins (PBPs) known to be implicated in the last stages of cell wall biosynthesis. PBPs constitute a group of membrane-associated proteins that catalyse the final reactions of cell wall assembly, namely the PG transglycosylation and transpeptidation/carboxypeptidation reactions. PBPs are the targets for β-lactam antibiotics, which are structural analogues of the D-Ala–D-Ala stem peptide moiety of the PG precursor, resulting in blocking either the carboxypeptidase or transpeptidase (involving first a carboxypeptidation of the terminal D-Ala residues before transpeptidating with an amino acceptor group) activities. To further investigate the possibility of Orf18 altering these processes, we first examined whether this protein could interact with *L. lactis* PBPs (data not shown). Surprisingly, we discovered that Orf18 itself has the capacity to bind penicillin. Figure 11A illustrates the binding of penicillin to small amounts of Orf18 by following a procedure, which is commonly used for the characterization of PBPs. This new and unexpected result led us to hypothesize that in order to inhibit the PG synthesis locally, Orf18 inhibits PBPs' carboxypeptidase and/or transpeptidase activities by sequestering the substrate of the reaction, as observed for glycopeptide antibiotics such as vancomycin (for a review, see Mainardi *et al.*, 2008 [38]). We subsequently tested whether Orf18 could inhibit D-Ala–D-Ala carboxypeptidase activity. For that, the C-terminal part of the protein, including HCD and the CHAP domain, was mixed with a commercial carboxypeptidase and the activity of the enzyme was measured in the presence of the substrate, a peptide containing a carboxyterminal D-alanine. We observed that in the presence of Orf18, very little proteolytic activity occurred while our control samples (BSA or buffer) indicated strong D-Ala–D-Ala cleavage. Furthermore, the subsequent addition of an excess of substrate to the reaction mixture containing Orf18 as well as the carboxypeptidase resulted in efficient cleavage of the substrate (data not shown). This latter result confirms that Orf18 does not inhibit the reaction by directly binding to the carboxypeptidase, thus suggesting that Orf18 instead

binds to the peptidyl-D-Ala–D-Ala extremity of the substrate thereby blocking the carboxypeptidation reaction.

Construction of L. lactis strains expressing both Orf18 and the listerial endolysin

We used *L. lactis* strains that express a bacteriophage endolysin active against *Listeria monocytogenes* to demonstrate that orf18 expression can promote the release of a biotechnologically relevant heterologous protein that was expressed intracellularly. The endolysin expressing strains have been described previously by Payne et *al.* [12] and they have the *Listeria* endolysin gene integrated within the lactococcal chromosome under the control of the promoter of the lactose catabolic operon. Strains constructed for these experiments are described in Table 2. They contain a plasmid vector with 1) the entire *orf18* gene, 2) an *orf18* gene lacking the CHAP domain or 3) no *orf18* gene. Also they all express the LM-4 lysin gene under the control of the *lac* promoter [12]. Recombinant strains were obtained using the strain FI7800 containing the LM-4 lysin gene integrated into the *lacG* gene of the lactose operon [12] as a donor strain in a conjugation experiment with the sex factor negative recipient strains FI10703, FI10704 and FI10705. These three strains carry in their chromosome the nisin-sucrose conjugative transposon Tn*5307* with an inactivated *nisA* gene [24] allowing controlled gene expression from the P_{nisA} promoter with externally added nisin. Conjugation experiments were performed as previously described by Stentz *et al.* [5] with donor and recipient mixtures grown on non-selective medium for 16 hours prior to selection of transconjugants on McKays indicator plates [25] containing lactose and the appropriate antibiotics. The selected transconjugants lost their ability to metabolise sucrose. Since the genes involved in sucrose metabolism are associated to the Tn*5307* nisin transposon, the transfer of the lactose operon via the sex factor from the donor strain led to a loss of the nisin transposon originally located into the recipient strain chromosome. For each strain, the nisin transposon was reintroduced by conjugation with the sex factor negative donor FI9979. The resultant strains FI10717, FI10718 and FI10719 were assessed for their ability to release the LM-4 endolysin following nisin induction.

Orf18 expression leads to listerial endolysin release

Overnight cultures of the strains FI10717, FI10718 and FI10719 (Table 2) expressing the *Listeria* LM-4 lysin under the P_{lac} promoter and the expression vector pUK200, pUK200 containing *orf18* and pUK200 containing *orf18* lacking the CHAP domain, respectively,

were diluted 100 times in fresh M17 buffered with potassium phosphate (0.2M, pH7). The cells were grown to an OD_{600} of 0.6 and the culture medium was split into two sets of parallel samples, control cultures and cultures in which 10 ng mL^{-1} of nisin was added to induce *orf18* expression. The cells were grown for 16 hours and the supernatant of these cultures was used neat or concentrated 5 times by filtration using a Vivaspin 6 mL concentrator column (MW 5000, Sartorius). 10 µL of the different supernatants was loaded into wells formed in a 1% agar in potassium phosphate buffer (0.2M, pH7) layer inoculated with 1:100 dilution of autoclaved *Listeria monocytogenes* FI6868 strain [12]. The agar plate was incubated for 24 hours at 30°C and observed for lytic zones. No lytic zone could be observed for FI10717 supernatants obtained from the control *L. lactis* strain that do not express Orf18. However, clear lytic zones could be obtained for cells FI10718 and FI10719 (Figure 6), both expressing Orf18 showing that Orf18 expression facilitates Listeria cell lysis. The absence of lytic zone observed for FI10717 grown in the presence of nisin showed that nisin had no effect on the release of active protein into the medium.

We confirmed that the observed lytic zones are the result of the LM-4 lysin activity released into the *L. lactis* culture medium and not Orf18 activity. Strain FI10704 expressing Orf18 but not LM4 was grown in the conditions used for FI10717, FI10718 and FI10719 and the neat culture supernatant and the 5 times concentrated supernatant were loaded on the same plate (Figure 6). No lytic zone was formed, indicating that LM4 only is responsible for the lysis of *Listeria* cells. It is noticeable that in the absence of the inducer nisin, FI10718 and FI10719 still produced a small lytic zone and this is due to the basal activity of the P_{nisA} promoter that has previously been described [26].

We have shown that the C-terminal CHAP domain was not necessary to promote cell lysis in cells expressing Orf18. In accordance with these results, we show that cells expressing Orf18 truncated in its CHAP domain produced lytic zones comparable to the wild-type Orf18, confirming that the CHAP domain is not involved in cell lysis of cells expressing Orf18.

These results demonstrate that Orf18 is able to release intracellular material by controlled lysis without the need for the use of an external lytic agent.

Demonstration of release of a model GI tract relevant therapeutic protein by controlled lysis

In order to establish the wider potential of the Orf18 release concept we made a new construct in which the heterologous protein is a GI tract relevant therapeutic. We expressed a synthetic gene for the human form of cytokine IL-10 (optimised for lactococcal codon usage) under lactose promoter control. The gene replacement technology used in this study was derived from the gene replacement technology used to construct *L. lactis Listeria* endolysin-expressing strains [12]. Instead of using a suicide vector, a plasmid based on the pGhost thermosensitive vector [32] was constructed (Fernandez, pers. com.). This plasmid allows the integration into the *lacG* gene of the *lac* operon of heterologous genes under the control of the *nisA* gene translation signals for optimal expression. The nisin-sucrose conjugative transposon Tn*5307* for nisin induction [24] was introduced into the new IL-10 strain by conjugation. The control vector pUK200 and the *orf18*-expressing vector pFI2640 were used to transform the selected transconjugant.

The release of biologically active cytokine in response to Orf18 expression was evaluated using a commercial ELISA test (Table 8). Unlike the LDH release results (Table 5), no reduction in the amount of released protein is observed for nisin concentrations > 1 ng/ml, although this may reflect cell recovery after a longer period of growth in the presence of nisin (16 hours vs. 2 hours for the LDH study). Alternatively, the lower activity of LDH detected could be the result of negative regulation of LDH in cells whose metabolic activity is dramatically reduced. The maximum amount of IL-10 obtained in this study (47.2 pg/10^9 cells) is 100 times lower than the amount obtained for the secreted version of IL10 [33]. This can be attributed to more efficient release of the secreted version of IL-10 combined with the use of a stronger promoter. However, in therapeutic use, more modest expression might be advantageous.

Table 8

Evaluation of IL-10 release

Strain	Nisin (ng/ml)	OD_{600}	IL-10 (pg/10^9 cells)
vector	0	2.0	1.0
Orf18	0	2.2	0.9
Vector	1	2.3	5.41
Orf18	1	1.8	21.8
Vector	5	2.1	5.27
Orf18	5	1.3	47.2

In this study, the cells were grown for 16 hours after addition of nisin at an OD_{600} = 0.5. The final OD_{600} is indicated. The cell culture supernatant was collected. The release of biologically active cytokine in response to Orf18 expression was evaluated using IL10 ELISA kit (Biosource, Invitrogen). The values are the means from triplicate samples (standard deviation, ≤ 12% for OD600 and ≤ 5% for IL-10 quantification).

Development of non-recombinant over-expression of Orf18 and its impact on lactic starter cultures

The *orf18* gene is part of a large operon and it is expressed at a very low level under normal circumstances. As a result of other work we have described the co-integration of the sex factor with an autonomously replicating lactose plasmid [7] and have shown that this can elevate expression of another gene (*cluA*) that is part of the same operon [34]. These DNA rearrangements involve non-recombinant and naturally occurring phenomena and we investigate here their potential to elevate *orf18* expression and enhance intracellular enzyme release. For this, we measured the release of LDH into the medium from a strain harbouring the co-integrate lactose::sex factor plasmid. Quantitative RT-PCR experiments showed that the amount of *orf18* RNA transcript was 10 times higher in MG1827 containing the co-integrate plasmid than in the parental strain MG1363 containing the chromosomally-located sex factor. LDH release into the medium was measured for both strains after 16 hours of growth. The activity measured in MG1827 (18.19 +/- 2.7 IU/L) was 8-fold higher than in MG1629 (2.2 +/- 0.6 IU/L) and this is in accordance with the results of LDH release measured in section 1 for an induction with 1ng/ml of nisin.

Conclusions

The results presented here show that Orf18 encoded by the *L. lactis* sex factor has, when overexpressed, the faculty to interfere with septum synthesis and to weaken the newly synthesized cell wall structure, generating cells unable to divide and susceptible to lysis. β-Lactams such as ampicillin are known to inhibit bacterial cell division [59]. After addition of ampicillin to a culture of *E. coli*, the cell density first doubles and then decreases rapidly due to sudden cell lysis [60]. Similarly, we show in this study that *L. lactis* cells treated with ampicillin or overexpressing Orf18 grow until their cell density doubles. At this point, addition of detergent is necessary to observe cell lysis due to the greater thickness of the cell wall in Gram positive bacteria than in Gram-negative bacteria. Another common feature with ampicillin-treated *E. coli* cells is the observed changes in cell morphology. Microscope observations of ampicillin-treated *E. coli* cells have shown prominent bulges at or near the middle of the cells as seen here for *L. lactis* cells when treated with ampicillin or overexpressing Orf18 (Fig. 8C). Functional analysis of the VirB1 orthologue protein P19 of plasmid R1 showed that its overexpression in *E. coli* led to the formation of vesicles protruding from the cell surface that were uniformly distributed on the surface [61]. In contrast, Orf18 has a targeted effect on the septum and on the newly synthesized cell wall of *L. lactis* (Fig. 8C). Assuming that all VirB1-like proteins described to date have a P19-like effect when overexpressed, this result demonstrates that Orf18 is the first T4SS-associated protein that generates such specific impacts on cell wall morphology. To further investigate the involvement of Orf18 in the inhibition of cell wall synthesis, we first examined the possibility for the protein to interact with PBPs as such proteins are known to be implicated in the last stages of PG biosynthesis. We anticipated that either the presence of Orf18 would affect the binding of penicillin to one or more PBPs, or that the binding of Orf18 to some PBPs would affect their electrophoretic migration. Surprisingly, we discovered that Orf18 itself has the capacity to bind penicillin (Fig. 11A). This new and unexpected result strongly suggests that in order to inhibit the PG synthesis locally, Orf18 prevents PBPs' transpeptidase action by sequestering the PG precursor in contrast to the action of penicillin. The inhibition of the transpeptidation reaction would prevent cell division and give rise to bulges at the cell surface as observed in cells overexpressing Orf18. We have also shown the ability of Orf18 to inhibit the enzymatic action of a PBP (Fig. 11B), and that Orf18 does not inhibit the reaction by directly binding to the enzyme, indicating that Orf18 instead binds to the peptidyl-D-Ala–D-Ala extremity of the substrate. The other T4SS

components, which had been shown to act at the cell wall level, are proteins such as VirB1 of *A. tumefaciens* (239 aa), VirB1 of *B. suis* (238 aa), AtlA (181 aa) of *N. gonorrhoeae* or Orf7 encoded by the Grampositive plasmid pIP501. These cell wall hydrolases are of a significantly smaller size than Orf18 (870 aa), suggesting that the N-terminal moiety of Orf18 could be involved in additional transfer functions. Orf18 is also likely to interact with other T4SS proteins of the sex factor, as shown for VirB1-like components in different conjugative systems [62, 63, 64]. This is the first report of a T4SS protein involved in the cell wall-spanning of the translocation machinery that also impacts drastically on the conjugation capacity of bacterial cells. For instance, deletion of VirB1 in *A. tumefaciens* only results in a 10- to 100-fold reduction in DNA transfer efficiency [65, 66]; whereas a deletion of Orf18 leads to complete loss of sex factor DNA transfer (Fig. 5A). Our results also indicate that a combination of the HCD and the CHAP domain activities is necessary to create an efficient transport complex. We have shown that in addition to the important role of the HCD (Table 7), the CHAP domain of Orf18 is essential for high-frequency conjugation (Table 6) and that it exhibits some growth-independent lytic activity detectable after a long period of Orf18 overexpression (Fig. 10B). We propose that the combination of two distinct activities promoted by the HCD and the CHAP domain, such as local cell wall synthesis inhibition and local cell wall degradation, respectively, enables the assembly of the T4SS across the cell wall. In this work, we revealed the existence of a close relationship between DNA transfer machineries encoded by the sex factor of *L. lactis*, plasmids pCF10 and pTEF2 of *Enterococcus*, the Tn*5252*-like copies found in *S. agalactiae* 2603V/R, *S. suis* 05ZYH33 and 98HAH33 genomes and the two GIs X and XII of *S. agalactiae* NEM316 (Fig. 2 and Fig. 9). We note that these closely related systems are found exclusively in completely sequenced genomes of ovoid-shaped cocci that share a common shape and the same mode of division [67]. The relationship between these systems is supported by previous evidence. For example, the lactococcal LI.ltrB group II intron maturase gene is able to insert into a conserved target in other Grampositive relaxase genes [68]. In particular, the authors showed that an LL.ltrB derivative could move *in vivo* from a donor plasmid into the *pcfG* relaxase gene of the *E. faecalis* pCF10 plasmid, in which the insertion occurred at a precise target site. In a different work, Staddon *et al.* (2006) [69] showed that plasmids containing the origin of transfer *oriT* of pCF10 could be efficiently mobilized from *E. faecalis* to *L. lactis* or *S. agalactiae* in the presence of pCF10 transfer functions, indicating that the mating pore assembled by pCF10 is compatible to *L. lactis* and *S. agalactiae* species. Moreover, when the Orf18 enterococcal homologue B0020 is expressed in *L. lactis*, the cells are subject to lysis when exposed to SDS (R. Stentz,

unpubl. data). This result indicates that the HCD of B0020 has the capacity to affect lactococcal PG, and we speculate that the HCDs derived from the different orthologues will affect the cell wall structure of any of the three ovoid-shaped species. Comparison of the gene organization combined with phylogenetic studies of the proteins from the conjugation systems found in the three cocci species enabled us to define three distinct phylogenetic groups (Fig. 9). The first group includes the *Lactococcus* sex factor, the enterococcal plasmids pCF10 and pTEF2, and the GI X of *S. agalactiae* NEM316. The second group is constituted of the streptococcal Tn*5252*-like elements and the only representative of the third group is the GI XII of *S. agalactiae* NEM316. The identification of 10 conserved genes among the six genomes suggests that this gene cluster is the minimal set of genes required for the assembly of a functional DNA transfer machinery (*cluA*, *orf15*, *17*, *18 (csiA)*, *20*, *24*, *traD*, *orf28*, *34* and *mobA*).

Table 9

L. lactis subsp. *cremoris* strains and plasmids used

Strain	Relevant characteristics*	Source or reference
FI9979	Transconjugant in FI9012 background, sf neg, *nisA*$^-$, *suc*$^+$, StrR RifR	This work & Stentz *et al.* (2004)
FI10703	FI9979pUK200, CamR, sf neg, *nisA*$^-$, *suc*$^+$, StrR RifR	This work & Stentz *et al.* (2009)
FI10704	FI9979pFI2640, CamR, sf neg, *nisA*$^-$, *suc*$^+$, StrR RifR	This work & Stentz *et al.* (2009)
FI10705	FI9979pFI2641, CamR, sf neg, *nisA*$^-$, *suc*$^+$, StrR RifR	This work
MG5267	Lac+, Single copy of Lac operon in chromosome	[70]
FI10799	MG5267 containing the human IL-10 gene integrated into *lacG* of the *lac* operon, *nisA*$^-$, *suc*$^+$pFI2640, CamR	This work
MG1363	Plasmid-free derivative of NCDO 712	Gasson (1983)
MG1629	MG1363 containing the lactose plasmid pLP712	Gasson (1983)
MG1827	MG1363 with co-integrate lactose sex factor plasmid pMG827	Gasson *et al.*, (1992)

Plasmids	Relevant characteristics*	Source or reference
pUK200	CamR, *PnisA*, pSH71 replicon	Wegmann *et al.* (1999)
pFI2640	pUK200, *PnisA*::*csiA*	This work & Stentz *et al.* (2009)

Plasmids	Relevant characteristics*	Source or reference
pFI2641	pUK200, *PnisA::csiAΔCHAP*	This work & Stentz *et al.* (2009)
pFI2649	pGhost-based vector containing *lacG* flanking sequences for gene integration into *L. lactis lac* operon. The integrated gene is fused to the *nisA* gene translation signals.	Fernandez (pers. com.)
pTG262	CamR, pSH71 replicon	Gasson and Anderson (1985)

* Cam = chloramphenicol; Str = streptomycin; Rif = rifampicin; sf neg = sex factor negative; *nisA*$^-$ = *nisA* negative ; *suc*$^+$ = sucrose positive; R = resistant

REFERENCES

[1] Gasson, M.J. (1983) Journal of Bacteriology 154: 1-9.

[2] Godon, J.-J., Jury, K., Shearman, C.A., & Gasson, M.J. (1994) Molecular Microbiology 12: 655-663.

[3] Gasson, M.J., Godon, J.-J., Pillidge, C.J., Eaton, T.J., Jury, K. & Shearman, C.A. (1995) International Dairy Journal 4: 757-762.

[4] Stentz, R.1, Jury, K., Eaton, T. J., Parker, M. L., Narbad, A., Gasson, M. J. & Shearman, C. (2004) Microbiology 150: 2503-2512.

[5] Stentz, R., Gasson, M. & Shearman, C. (2006) Journal of Bacteriology 188: 2106-2114.

[6] Shearman, C.A., Godon, J.-J. & Gasson,M.J. (1996) Molecular Microbiology 21: 45-53.

[7] Gasson, M.J., Swindell, S., Maeda, S. & Dodd, H.M. (1992) Molecular Microbiology 6: 3213-3223.

[8] Wegmann, U., O'Connell-Motherway, M., Zomer, A., Buist, G., Shearman, *et al.* (2007) Journal of Bacteriology 189: 3256-3270.

[9] smart search results

[10] Donovan, D.M., Foster-Frey, J., Dong, S., Rousseau, G.M., Moineau, S. & Pritchard, D.G. (2006) Appl. Envir. Microbiol. 72: 5108-5112.

[11] Gasson (1995-2003) Patents GB 2255561 B (1995); AU 650737B (1994); US 5763251 (1998); US 6083684 (2000); CA 2066387 (2003); EP 0510907B (2003).

[12] Payne,J., MacCormick, C.A., Griffin, H.G. & Gasson, M.J. (1996) FEMS Microbiology Letters 136: 19-24.

[13] Steidler, L., W. Hans, L. Schotte, S. Neirynck, F. Obermeier, W. Falk, W. Fiers, and E. Remaut. (2000) Science 289:1352-1355.

[14] Braat, H., Rottiers, P., Hommes, D.W., Huyghebaert, N., *et al.* (2006) Clinical Gastroenterology and Hepatology 4: 754-759.

[15] Steidler, L & Rottiers, P. (2006) Annals of the New York Academy of Sciences 1072: 176-186.

[16] Shearman, C.A., Jury, K. & Gasson, M.J. (1992) Biotechnology 10: 196-199

[17] Sambrook and Russel, 2001)

[18] Terzaghi and Sandine (1975) *Appl. Microbiol.* **29,** 807-813.

[19] Dower, W.J., Miler, J.F. & Ragsdale, C.W. (1988) Nucleic Acids Research 16: 6127-6145.

[20] Holo, H. & Nes, L.F. (1995) Methods Mol. Biol. 47: 195-199.

[21] Gibson, T.J. (1984) Ph.D. Thesis Cambridge University, Cambridge, UK.

[22] Link, A.J., Phillips, D. & Church, G.M. (1997) J. Bacteriol. 179: 6228-6237.

[23] Leenhouts, K., Buist, G., Bolhuis, A. Ten Berge, A., Kiel, J., Mierau, I., Dabrowska, M., Venema, G. & Kok, J. (1996) Mol. Gen. Genet. 253 : 217-224.

[24] Dodd, H.M., Horn, N. & Gasson, M.J. (1996) *Microbiology* **142:**2385-92.

[25] Kondo & McKay (1982) *Appl. Environ. Microbiol.* **43,** 1213-1215.

[26] De Ruyter, P.G., Kuipers, O.P., & De Vos, W.M. (1996) Appl. Environ. Microbiol. 62 : 3662-3667.

[27] Wegmann, U., Klein, J.R., Drumm, Kuipers, O.P. & Henrich, B. (1999) Appl. Environ. Microbiol. 65 : 4729-4733.

[28] Gasson, M.J. and Anderson, P.H. (1985) High copy number plasmid vectors for use in lactic streptococci. *FEMS Microbiology Letters* **30:**193–196.

[29] Horn, N., Wegmann, U., Narbad, A., and Gasson, M.J. (2005) Characterisation of a novel plasmid p9785S from *Lactobacillus johnsonii* FI9785. *Plasmid* **54:**176–183.

[30] Kleerebezem, M., Beerthuyzen, M.M., Vaughan, E.E., de Vos, W.M., and Kuipers, O.P. (1997) Controlled gene expression systems for lactic acid bacteria: transferable nisin-inducible expression cassettes for Lactococcus, Leuconostoc, and Lactobacillus spp. *Appl Environ Microbiol* **63:**4581-4584.

[31] MacCormick, C.A., Griffin, H.G. and Gasson, M.J. (1995) Construction of a food-grade host/vector system for *Lactococcus lactis* based on the lactose operon. *FEMS Microbiol Lett* **127:**105-109.

[32] Maguin, E., Duwat, P., Hege, T., Ehrlich, D., and Gruss, A. (1992) New thermosensitive plasmid for gram-positive bacteria. *J Bacteriol* **174:**5633-5638.

[33] Steidler, L., Neirynck, S., Huyghebaert, N., Snoeck, V., Vermeire, A., Goddeeris, B., Cox, E., Remon J.P., and Remaut, E. (2003) Biological containment of genetically modified Lactococcus lactis for intestinal delivery of human interleukin 10. *Nat Biotechnol* **21:**785-9.

[34] Stentz, R., Wegmann, U., Parker, M., Bongaerts, R., Lesaint, L., Gasson, M. and Shearman, C. (2009) CsiA is a bacterial cell wall synthesis inhibitor contributing to DNA translocation through the cell envelope. *Mol Microbiol* **72:**779-794.

[35] Dunny, G., Funk, C., and Adsit, J. (1981) Direct stimulation of the transfer of antibiotic resistance by sex pheromones in *Streptococcus faecalis. Plasmid* **6:** 270–278.

[36] Hirt, H., Schlievert, P.M., and Dunny, G.M. (2002) *In vivo* induction of virulence and antibiotic resistance transfer in *Enterococcus faecalis* mediated by the sex pheromonesensing system of pCF10. *Infect Immun* **70:** 716–723.

[37] Herbert, M.A., Beveridge, C.J., McCormick, D., Aten, E., Jones, N., Snyder, L.A., and Saunders, N.J. (2005) Genetic islands of *Streptococcus agalactiae* strains NEM316 and 2603VR and their presence in other Group B streptococcal strains. *BMC Microbiol* **5:** 31.

[38] Mainardi, J.-L., Villet, R., Bugg, T.D., Mayer, C., and Arthur, M. (2008) Evolution of peptidoglycan biosynthesis under the selective pressure of antibiotics in Gram-positive bacteria. FEMS Microbiol Rev **32**: 386–408.

[39] Frère, J.M., Klein, D., and Ghuysen, J.M. (1980) Enzymatic method for rapid and sensitive determination of betalactam antibiotics. Antimicrob Agents Chemother **18**: 506–510.

[40] Zhao, G., Meier, T.I., Kahl, S.D., Gee, K.R., and Blaszczak, L.C. (1999) BOCILLIN FL, a sensitive and commercially available reagent for detection of penicillin-binding proteins. Antimicrob Agents Chemother 43: 1124–1128.

[41] Mulder, N.J., Apweiler, R., Attwood, T.K., Bairoch, A., Bateman, A., Binns, D., *et al.* (2007) New developments in the InterPro database. *Nucleic Acids Res* **35:** D224–D228.

[42] Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E.L. (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* **305:** 567–580.

[43] Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., *et al.* (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23:** 2947–2948.

[44] Stavrinides, J., and Guttman, D.S. (2004) Nucleotide sequence and evolution of the five-plasmid complement of the phytopathogen *Pseudomonas syringae* pv. Maculicola ES4326. *J Bacteriol* **186:** 5101–5115.

[45] Paulsen, I.T., Banerjei, L., Myers, G.S.A., Nelson, K.E., Seshadri, R., Read, T.D., *et al.* (2003) Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. *Science* **299:** 2071–2074.

[46] Ayoubi, P., Kilic, A.O., and Vijayakumar, M.N. (1991) Tn*5253*, the pneumococcal omega (cat tet) BM6001 element, is a composite structure of two conjugative transposons, Tn*5251* and Tn*5252*. *J Bacteriol* **173:** 1617–1622.

[47] Tettelin, H., Masignani, V., Cieslewicz, M.J., Donati, C., Medini, D., Ward, N.L., *et al.* (2002) Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V. *Streptococcus agalactiae*. *Proc Natl Acad Sci USA* **99:** 12391–12396.

[48] Chen, C., Tang, J., Dong, W., Wang, C., Feng, Y., Wang, J., *et al.* (2007) A glimpse of streptococcal toxic shock syndrome from comparative genomics of *S. suis* 2 Chinese isolates. *PLoS ONE* **2:** e315.

[49] Glaser, P., Rusniok, C., Buchrieser, C., Chevalier, F., Frangeul, L., Msadek, T., *et al.* (2002) Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. *Mol Microbiol* **45:** 1499–1513.

[50] Hirt, H., Manias, D.A., Bryan, E.M., Klein, J.R., Marklund, J.K., Staddon, J.H., *et al.* (2005) Characterization of the pheromone response of the *Enterococcus faecalis* conjugative plasmid pCF10: complete sequence and comparative analysis of the transcriptional and phenotypic responses of pCF10-containing cells to pheromone induction. *J Bacteriol* **187:** 1044–1054.

[51] Olmsted, S.B., Kao, S.M., van Putte, L.J., Gallo, J.C., and Dunny, G.M. (1991) Role of the pheromone inducible surface protein Asc10 in mating aggregate formation and conjugal transfer of the *Enterococcus faecalis* plasmid pCF10. *J Bacteriol* **173:** 7665–7672.

[52] Demuth, D.R., Golug, E.E., and Malamud, D. (1990) Streptococcal–host interactions. Structural and functional analysis of a *Streptococcus sanguis* receptor for a human salivary glycoprotein. *J Biol Chem* **265:** 7120–7126.

[53] Mills, D.A., McKay, L.L., and Dunny, G.M. (1996) Splicing of a group II intron involved in the conjugative transfer of pRS01 in lactococci. *J Bacteriol* **178:** 3531–3538.

[54] Le Bourgeois, P., Daveran-Mingot, M.L., and Ritzenthaler, P. (2000) Genome plasticity among related *Lactococcus* strains: identification of genetic events associated with macrorestriction polymorphisms. *J Bacteriol* **182:** 2481– 2491.

[55] Brown, N.F., Wickham, M.E., Coombes, B.K., and Finlay, B.B. (2006) Crossing the line: selection and evolution of virulence traits. *PLoS Pathog* **2:** e42.

[56] Kuipers, O.P., de Ruyter, P.G., Kleerebezem, M., and de Vos, W.M. (1997) Controlled overproduction of proteins by lactic acid bacteria. *Trends Biotechnol* **15:** 135–140.

[57] Lewington, J., Greenaway, S.D., and Spillane, B.J. (1987) Rapid small-scale preparation of bacterial genomic DNA, suitable for cloning and hybridization analysis. *Lett Appl Microbiol* **5:** 51–53.

[58] Ciak, J., and Hahn, F.E. (1957) Penicillin-induced lysis of *Escherichia coli. Science* **125:** 119–120.

[59] Schwarz, U., Asmus, A., and Frank, H. (1969) Autolytic enzymes and cell division of *Escherichia coli. J Mol Biol* **41:** 419–429.

[60] Burdett, I.D., and Murray, R.G. (1974) Septum formation in *Escherichia coli*: characterization of septal structure and the effects of antibiotics on cell division. *J Bacteriol* **119:** 303–324.

[61] Bayer, M., Iberer, R., Bischof, K., Rassi, E., Stabentheiner, E., Zellnig, G., and Koraimann, G. (2001) Functional and mutational analysis of p19, a DNA transfer protein with muramidase activity. *J Bacteriol* **183:** 3176–3183.

[62] Ward, D.V., Draper, O., Zupan, J.R., and Zambryski, P.C. (2002) Peptide linkage mapping of the *Agrobacterium tumefaciens vir*-encoded type IV secretion system reveals protein subassemblies. *Proc Natl Acad Sci USA* **99:** 11493–11500.

[63] Höppner, C., Carle, A., Sivanesan, D., Hoeppner, S., and Baron, C. (2005) The putative lytic transglycosylase VirB1 from *Brucella suis* interacts with the type IV secretion system core components VirB8, VirB9 and VirB11. *Microbiology* **151:** 3469–3482.

[64] Abajy, M.Y., Kopec´, J., Schiwon, K., Burzynski, M., Döring, M., Bohn, C., and Grohmann, E. (2007) A type IV-secretion-like system is required for conjugative DNA transport of broad-host-range plasmid pIP501 in Grampositive bacteria. *J Bacteriol* **189:** 2487–2496.

[65] Berger, B.R., and Christie, P.J. (1994) Genetic complementation analysis of the *Agrobacterium tumefaciens virB* operon: *virB2* through *virB11* are essential virulence genes. *J Bacteriol* **176:** 3646–3660.

[66] Bohne, J., Yim, A., and Binns, A.N. (1998) The Ti plasmid increases the efficiency of *Agrobacterium tumefaciens* as a recipient in *virB*-mediated conjugal transfer of an IncQ plasmid. *Proc Natl Acad Sci USA* **95:** 7057–7062.

[67] Zapun, A., Vernet, T., and Pinho, M.G. (2008) The different shapes of cocci. *FEMS Microbiol Rev* **32:** 345–360.

[68] Staddon, J.H., Bryan, E.M., Manias, D.A., and Dunny, G.M. (2004) Conserved target for group II intron insertion in relaxase genes of conjugative elements of Gram-positive bacteria. *J Bacteriol* **186:** 2393–2401.

[69] Staddon, J.H., Bryan, E.M., Manias, D.A., Chen, Y., and Dunny, G.M. (2006) Genetic characterization of the conjugative DNA processing system of enterococcal plasmid pCF10. *Plasmid* **56:** 102–111.

[70] MacCormick CA, Griffin HG, Gasson MJ. (1995) Construction of a food-grade host/vector system for Lactococcus lactis based on the lactose operon. *FEMS Microbiol Lett.*, **127**(1-2):105-9.

[71] Godon, J.-J., Jury, K., Shearman, C.A., and Gasson, M.J. (1994) The *Lactococcus lactis* sex-factor aggregation gene. *Clua Mol Microbiol* **12:** 655–663.

[72] Shearman, C.A., Godon, J.-J., and Gasson, M. (1996) Splicing of a group II intron in a functional transfer gene of *Lactococcus lactis*. *Mol Microbiol* **21:** 45–53.

CLAIMS

1. A bacterial host cell having improved cell permeability properties, the cell comprising an *Orf18* gene or species homologue thereof, or a fragment or variant of the same encoding a polypeptide having the activity of the *Orf18* gene product or species homologue thereof, wherein the gene, homologue, fragment or variant is under the control of an heterologous promoter which permits sufficient expression of the gene, homologue, fragment or variant to increase the permeability of the cell wall.

2. A host cell according to Claim 1 wherein the cell is capable of inducible cell lysis.

3. A host cell according to Claim 1 or 2 wherein the cell wall is more permeable than a corresponding host cell lacking the *Orf18* gene, homologue, fragment or variant thereof.

4. A host cell according to Claim 1 or 2 wherein a chromosomally-integrated *Orf18* gene naturally present in the bacterial cell is inactivated.

5. A host cell according to Claim 4 wherein the chromosomally-integrated *Orf18* gene naturally present in the bacterial cell is deleted, at least in part.

6. A host cell according to any one of the preceding claims where the cell is a Gram-positive bacterial cell.

7. A host cell according to Claim 6 wherein the cell is selected from the group consisting of *Lactococcus* cells, *Lactobacillus* cells, *Bacillus subtilis* cells and *Clostridium* cells.

8. A host cell according to Claim 7 wherein the cell is a *Lactococcus* cell.

9. A host cell according to Claim 8 wherein the *Lactococcus* cell is selected from the group consisting of NCIMB8662 (=HP), NCIMB700499 (=UD459), NCIMB700500 (=UD496), NCIMB700504 (=UD806), NCIMB700508 (=C7), NCIMB700562 (=D31), NCIMB700609 (=RW), NCIMB700762 (=ML1), NCIMB70216 (=SK11), NCIMB700278 (=FD50) and NCIMB7012008 (=TR).

10. A host cell according to Claim 8 or 9 wherein the cell is a *Lactococcus lactis* cell.

11. A host cell according to Claim 10 wherein the cell is a *Lactococcus lactis* cells of strain MG1363.

12. A host cell according to Claim 7 wherein the cell is a *Lactobacillus* cell.

13. A host cell according to Claim 7 wherein the cell is a *Bacillus subtilis* cell.

14. A host cell according to Claim 7 wherein the cell is a *Clostridium* cell.

15. A host cell according to any one of the preceding claims where the cell is a Gram-negative bacterial cell.

16. A host cell according to Claim 15 wherein the cell is an *Escherichia coli* cell.

17. A host cell according to any one of the preceding claims wherein the *Orf18* gene, species homologue, fragment or variant thereof is located extra-chromosomally on a plasmid.

18. A host cell according to any one of Claims 1 to 16 wherein the *Orf18* gene, species homologue, fragment or variant thereof is chromosomally integrated.

19. A bacterial host cell according to Claim 18 wherein the host cell comprises a naturally-occurring chromosomal *Orf18* gene, or homologue thereof, under the control of an heterologous promoter.

20. A host cell according to any one of the preceding claims wherein the *Orf18* gene comprises the nucleotide sequence of SEQ ID NO:1.

21. A host cell according to any one of the preceding claims wherein the cell comprises an *Orf18* gene, or active fragment or variant thereof

22. A host cell according to Claim 21 wherein the cell comprises an *Orf18* gene, or active fragment thereof.

23. A host cell according to Claim 22 wherein the cell comprises an *Orf18* gene.

24. A host cell according to any one of Claims 21 or 23 wherein the *Orf18* gene comprises or consists of the nucleotide sequence of SEQ ID NO:1.

25. A host cell according to Claim 21 wherein the cell comprises a fragment of an *Orf18* gene.

26. A host cell according to Claim 25 wherein the *Orf18* gene fragment excludes a C-terminal cysteine, histidine-dependent aminohydrolase/peptidase domain (CHAP) domain of the *Orf18* gene.

27. A host cell according to any one of the preceding claims wherein the *Orf18* gene, homologue, fragment or variant is under the control of an inducible promoter.

28. A host cell according to Claim 27 wherein the inducible promoter is P_{nisA}.

29. A host cell according to Claim 27 wherein the inducible promoter is induced by a carbon source selected from the group consisting of xylose, lactose and sucrose.

30. A host cell according to Claim 27 wherein the inducible promoter is a late/stationary phase promoter.

31. A host cell according to any one of Claims 1 to 26 wherein the *Orf18* gene, homologue, fragment or variant is under the control of a constitutive promoter.

32. A host cell according to Claim 31 wherein the cell is viable.

33. A host cell according to any one of the preceding claims wherein the host cell further comprises a polypeptide for release, and/or a nucleic acid molecule encoding the same.

34. A host cell according to Claim 33 wherein the polypeptide for release is a bioactive polypeptide.

35. A host cell according to Claim 34 wherein the bioactive polypeptide is selected from the group consisting of vaccine antigens, immune modulators, antimicrobial agents, anti-angiogenic agents and growth factors.

36. A host cell according to Claim 34 or 35 wherein the bioactive polypeptide is an interleukin, for example interleukin-10 or interleukin-12.

37. A host cell according to Claim 34 or 35 wherein the bioactive polypeptide has efficacy in the treatment of inflammatory bowel disorder, for example interleukin-10, keratinocyte growth factor (KGF), trefoil factor (TFF) or transforming growth factor (TGF)-β.

38. A host cell according to Claim 34 or 35 wherein the bioactive polypeptide has efficacy in the treatment of colon cancer, for example endostatin or soluble vascular endothelial growth factor receptor VEGFR-1.

39. A host cell according to Claim 33 wherein the polypeptide for release is an enzyme for industrial or domestic use.

40. A host cell according to Claim 39 wherein the polypeptide for release is selected from the group consisting of peptidases, proteinases, esterases, lipases and endolysins.

41. A host cell according to Claim 40 wherein the polypeptide is an endolysin.

42. A host cell according to Claim 41 wherein the endolysin is selected from a group consisting of endolysins that target *Streptococcus pneumoniae* (for example Pal, Cpl-1 and LytA), endolysins that target *Clostridium perfringens* (for example, the lysin of bacteriophage ϕ3626), endolysins that target *Bacillus anthracis* / *Bacillus cereus* (for example, endolysin PlyG), endolysins that target *Staphylococcus aureus* (for example, endolysins phi11 and phi 12) and endolysins that target *Enterococcus faecalis* and *faecium* (for example, endolysin PlyV12).

43. A host cell according to Claim 41 or 42 wherein the polypeptide for release is a bacteriophage endolysin.

44. A host cell according to Claim 43 wherein the bacteriophage endolysin is an endolysin of a bacteriophage selected from the group consisting of bacteriophage ΦCD27 of *Clostridium difficile*, bacteriophage ΦP1 of *Clostridium tyrobutyricum* and bacteriophage ΦLM4 of *Listeria monocytogenes*.

45. A host cell according to any one of the preceding claims comprising a species homologue of an *Orf18* gene, or a fragment or variant of the same, encoding a polypeptide having the activity of the *Orf18* gene product.

46. A host cell according to Claim 45 wherein the species homologue is selected from the group consisting of the *B0020* gene of conjugative plasmid pTEF2 of *Enterococcus faecalis*, the *prgK* gene of conjugative plasmid pCF10 of *Enterococcus faecalis*, the *SAG1286* gene of conjugative transposon Tn5252 of *Streptococcus agalactiae* and the *gbs1133* and *gbs1359* genes of *Streptococcus agalactiae* strain NEM316.

47. A kit for use in the production of a recombinant protein comprising:

(a) a bacterial cell capable of exhibiting improved cell permeability properties upon transformation with a plasmid comprising an *Orf18* gene or species homologue thereof, or a fragment or variant of the same encoding a polypeptide having the activity of the *Orf18* gene product or species homologue thereof, wherein the gene, homologue, fragment or variant is under the control of an heterologous promoter which permits sufficient expression of the gene, homologue, fragment or variant to increase the permeability of the cell; and

(b) a plasmid comprising an *Orf18* gene or species homologue thereof, or a fragment or variant of the same encoding a polypeptide having the activity of the *Orf18* gene product or species homologue thereof, wherein the gene, homologue, fragment or variant is under the control of an heterologous promoter which permits sufficient expression of the gene, homologue, fragment or variant to increase the permeability of the cell.

48. A kit according to Claim 44 wherein the bacterial cell is a cell as defined in any one of Claims 2 to 16 or 33 to 44.

49. A kit according to Claim 32 or 33 wherein the *Orf18* gene, homologue, fragment or variant thereof is a gene, homologue, fragment or variant as defined in any one of Claims 20 to 32, 45 or 46.

50. A kit according to any one of Claims 47 to 49 comprising a host cell according to any one of Claims 1 to 46.

51. A kit according to any one of Claims 47 to 50 further comprising an inducer for inducing expression of the *Orf18* gene, homologue, fragment or variant thereof.

52. A kit according to Claim 51 wherein the inducible promoter is P_{nisA} and the inducer is nisin.

53. A kit according to any one of Claims 47 to 52 further comprising one or more reagents or media for cell culture.

54. An isolated nucleic acid molecule comprising an *Orf18* gene or species homologue thereof, or a fragment or variant of the same encoding a polypeptide having the activity of the *Orf18* gene product or species homologue thereof, wherein the gene, homologue, fragment or variant is under the control of an heterologous promoter which permits sufficient expression of the gene, homologue, fragment or variant to increase the permeability of the cell.

55. An isolated nucleic acid molecule according to Claim 54 wherein the *Orf18* gene, homologue, fragment or variant thereof is a gene, homologue, fragment or variant as defined in any one of Claims 20 to 32, 45 or 46.

56. An isolated nucleic acid molecule according to Claim 54 or 55 comprising or consisting of DNA.

57. An isolated nucleic acid molecule according to any one of Claims 54 to 56 wherein the nucleic acid molecule is a plasmid.

58. Use of a host cell according to any one of Claims 1 to 46 in the production of a polypeptide.

59. A method for producing a polypeptide comprising culturing a host according to any one of Claims 33 to 44 under conditions which allow expression of the polypeptide for release.

60. A method according to Claim 59 further comprising permitting or enhancing release of the polypeptide from the host cells by exposing the cells to an inducer for inducing expression of the *Orf18* gene, homologue, fragment or variant thereof.

61. A method according to Claim 60 wherein the inducer is used in a concentration sufficient to lyse the cells.

62. A method according to Claim 60 wherein the inducer is used in a concentration sufficient to permit or enhance release of the polypeptide from the host cells but not enough to lyse the cells.

63. A pharmaceutical composition comprising a host cell according to any one of Claims 1 to 46.

64. A pharmaceutical composition according to Claim 63 for oral administration.

65. A host cell according to any one of Claims 1 to 46 for use in medicine.

66. A host cell according to any one of Claims 1 to 46 for administering a bioactive agent to the human or animal body.

67. A host cell according to Claim 66 for delivering a bioactive agent to the GI tract.

68. Use of a host cell according to any one of Claims 1 to 46 in the manufacture of a medicament for administering a bioactive agent to the human or animal body.

69. The use according to Claim 68 in the manufacture of a medicament for delivering a bioactive agent to the GI tract.

70. A method for administering a bioactive agent to the human or animal body comprising administering a host cell according to any one of Claims 1 to 46 or a pharmaceutical composition according to Claim 63 or 64.

71. A method according to Claim 70 for administering a bioactive agent to the GI tract.

72. Use of a host cell according to any one of Claims 1 to 46 in the production of a diary product.

73. A use according to Claim 72 wherein the dairy product is a cheese.

74. A method for producing a dairy product comprising exposing milk to a starter culture of host cells according to any one of Claims 1 to 46, wherein the host cells are lactic acid bacterial cells.

75. A method according to Claim 74 further comprising exposing the bacterial cells to an inducer to induce cell lysis (for example, following primary fermentation).

76. A method according to Claim 74 or 75 wherein the dairy product is a cheese.

77. A method of transforming a host cell according to any one of Claims 1 to 46 comprising exposing the cell to an inducer of expression of the *Orf18* gene or species homologue thereof, or a fragment or variant of the same, wherein the concentration of induce is sufficient to inhibit cell wall synthesis but not enough to lyse the cells.

78. A method according to Claim 77 wherein the cells are osmotically buffered.

79. A method according to Claim 77 further comprising electroporation of the cells.

80. A bacterial host cell substantially as described herein with reference to the description.

81. A kit substantially as described herein with reference to the description.

82. Use of a bacterial host cell for producing a polypeptide substantially as described herein with reference to the description.

83. A method for producing a polypeptide substantially as described herein with reference to the description.

84. A pharmaceutical composition substantially as described herein with reference to the description.

85. Use of a bacterial host cell in medicine substantially as described herein with reference to the description.

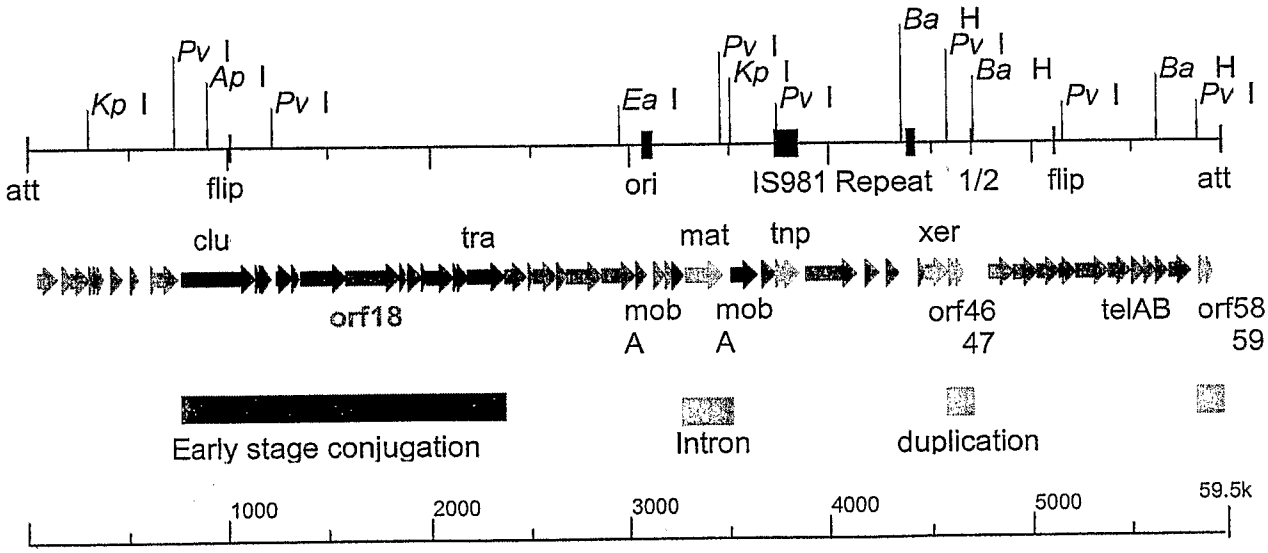
86. A method for administering a bioactive agent to the human or animal body substantially as described herein with reference to the description.

87. Use of a bacterial host cell in the production of a diary product substantially as described herein with reference to the description.

88. A method for producing a dairy product substantially as described herein with reference to the description.

89. A method of transforming a host cell substantially as described herein with reference to the description.

FIGURE 1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Pv I
Ap I
Pv I
Ea I
Pv I
Kp I
Pv I
Ba H
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Ba H
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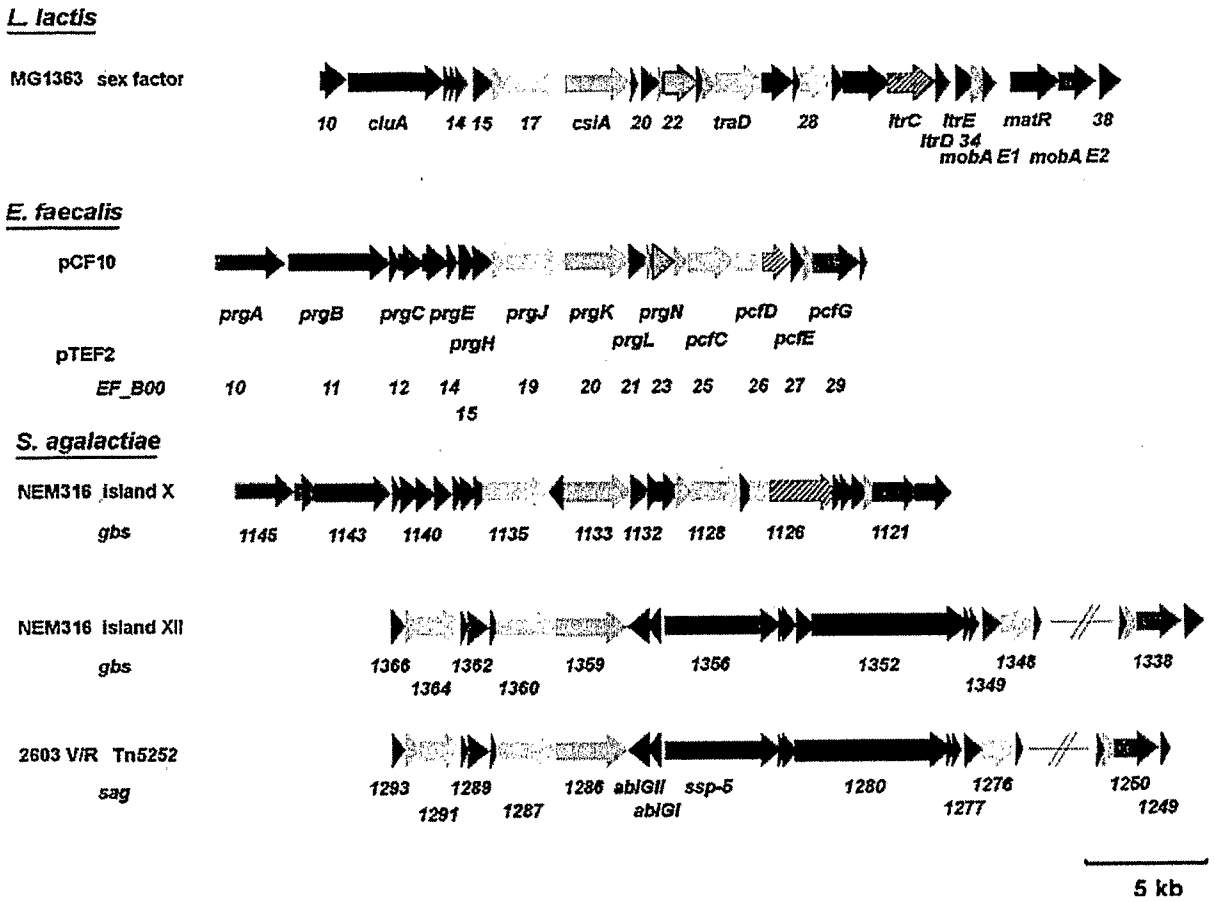
att
flip
ori
IS981 Repeat
1/2
flip
att
clu
tra
mat
tnp
xer
orf18
mob A
mob A
orf46 47
telAB
orf58 59
Early stage conjugation
Intron
duplication
1000
2000
3000
4000
5000
59.5k

FIGURE 2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MG1363 sex factor
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ltrD 34
mobA E1 mobA E2
E. faecalis
pCF10
prgA prgB prgC prgE prgJ prgK prgN pcfD pcfG
prgH prgL pcfC pcfE
pTEF2
EF_B00 10 11 12 14 19 20 21 23 25 26 27 29
15
S. agalactiae
NEM316 island X
gbs
1145 1143 1140 1135 1133 1132 1128 1126 1121
NEM316 island XII
gbs
1366 1362 1359 1356 1352 1348 1338
1364 1360 1349
2603 V/R Tn5252
sag
1293 1289 1286 ablGII ssp-5 1280 1276 1250
1291 1287 ablGI 1277 1249
5 kb

FIGURE 3

```
1     ATGGATAAAAGAAGCAAAGAAAACTTTAAAGCTTCGCTCAATGTCCAGAAACGTGTCTCTGATGGAATTGATGCGAATGCCTTTAGAAAA
1     M  D  K  R  S  K  E  N  F  K  A  S  L  N  V  Q  K  R  V  S  D  G  I  D  A  N  A  F  R  K
91    CCTCGACAAGCCACACAGCTTAGTAAGCAGACACTTAAAGAAAGCAAAAAAAATCTTAAAGTTCGGCGGACAAACTACAGGAAGCTTAAA
31    P  R  Q  A  T  Q  L  S  K  Q  T  L  K  E  S  K  K  N  L  K  V  R  R  T  N  Y  R  K  L  K
181   GCTGGTTCAGGTTTACAAGTTGTTGCAGCCAAAAAGAAATATCTTGAAGCAAAAGCTGATAAAAATGCTTCTAAAGTGGTTTTTAAAAAA
61    A  G  S  G  L  Q  V  V  A  A  K  K  K  Y  L  E  A  K  A  D  K  N  A  S  K  V  V  F  K  K
271   GCAAAGAAATCTGATCCAACACGAGCAGCAAACAAAGCGAAAGGCTATGCCAAGAATCAAGCCAAGCAAACAGCAGTCAACCACTTAGTG
91    A  K  K  S  D  P  T  R  A  A  N  K  A  K  G  Y  A  K  N  Q  A  K  Q  T  A  V  N  H  L  V
361   TTATCGCCTCTTGAAAAAGATGATACTTTAAGAGAAGGTTCAGACCTCTATAGAAAGACTCAACGTACCAAAAGTGGGTTAAAACTTTCT
121   L  S  P  L  E  K  D  D  T  L  R  E  G  S  D  L  Y  R  K  T  Q  R  T  K  S  G  L  K  L  S
451   AAGGATGTTGTCACTTCTTCCGCTAAAGCTTCGGTTAATCTTTCAAAGCATACTTACGGTTTAGCCAATCGAAGTGTTAATATGGTTCGA
151   K  D  V  V  T  S  S  A  K  A  S  V  N  L  S  K  H  T  Y  G  L  A  N  R  S  V  N  M  V  R
541   GGTCGTGGGTTTGTCAGAACTCCTTCTGAATTGGGTTTCAGAAGGCAAGCCGCAAAAAGAATGCGGAACTTTCAAAATAAGCTGAGTGCC
181   G  R  G  F  V  R  T  P  S  E  L  G  F  R  R  Q  A  A  K  R  M  R  N  F  Q  N  K  L  S  A
631   GCAAGAAAGGCAAAAAAGGCGGAGCAAGGATTTTCTTTGATTCGGTCAATCTTAAAAGGCCAACAAACCCTGAGTCGTGCGTTTACTTTA
211   A  R  K  A  K  K  A  E  Q  G  F  S  L  I  R  S  I  L  K  G  Q  Q  T  L  S  R  A  F  T  L
721   ATCGTGACCTCCAAAGGATTACTCGTCGTTCTTGCCGTTATGTTTATATTGTGTCTCTTGGGAATCCTTAACATGGCCTCCTCTGTTCCA
241   I  V  T  S  K  G  L  L  V  V  L  A  V  M  F  I  L  C  L  L  G  I  L  N  M  A  S  S  V  P
811   ACAAAGCAAGATGATTTTCAACTGACAAAGTCTTGGACGTATTTCACAAAACTTGATGCAGAACAATCAGAGGATGGAAATAACTTCTAC
271   T  K  Q  D  D  F  Q  L  T  K  S  W  T  Y  F  T  K  L  D  A  E  Q  S  E  D  G  N  N  F  Y
901   ACGCCTTTGGATGATGTGATGTTTTATATGAATGACCAATTTGAAGATTATAATTTACAAGATCAAGTGCCTGTAGGTTCAAATGGTGCA
301   T  P  L  D  D  V  M  F  Y  M  N  D  Q  F  E  D  Y  N  L  Q  D  Q  V  P  V  G  S  N  G  A
991   GCACTTCCGAACCAAAATTATGAACAATATCTTACAGGTCTTTGGTCAGCCTTAAATGGCTCTTCTCCTGATTATAAGCTAACAACAATG
331   A  L  P  N  Q  N  Y  E  Q  Y  L  T  G  L  W  S  A  L  N  G  S  S  P  D  Y  K  L  T  T  M
1081  GAGGCTTTGGAGACGGATAAGAGTTCTAAATATTATCTATCTCTTGATGATTATTCAGATTTTAAAGAACGTGTTAATGAAGTAGGTTAT
361   E  A  L  E  T  D  K  S  S  K  Y  Y  L  S  L  D  D  Y  S  D  F  K  E  R  V  N  E  V  G  Y
1171  GACAGCCTTGATGGTCAACTTCAATTTCCTTATCAAACAGAGAGTTTGGTTATCAATCGTAGATATGGCTACGAACGGAATGGAGAAAAA
391   D  S  L  D  G  Q  L  Q  F  P  Y  Q  T  E  S  L  V  I  N  R  R  Y  G  Y  E  R  N  G  E  K
1261  ACTGAACTCCACTCAAGCATTGATGTTAGTTCAGCACCAGGGCAAGATTTAACTTCACCAATGCACGGAATTGTTAATTCTGTGACTGAC
421   T  E  L  H  S  S  I  D  V  S  S  A  P  G  Q  D  L  T  S  P  M  H  G  I  V  N  S  V  T  D
1351  CCTAATACATTGGTCATTTCTGAAGCTGAAAATGCACGGCTGACAATTGTTGGGATTAATAGCGGACGGTTTATTGGAGGTGAAACAGTA
451   P  N  T  L  V  I  S  E  A  E  N  A  R  L  T  I  V  G  I  N  S  G  R  F  I  G  G  E  T  V
1441  GATGCTGGAACACTTCTTGGTAAGGCCACAAAGTCAAGTCTTAATATGACCTATGAAAAATATAATGAAGATGATAAAAAATGGGCAAAA
481   D  A  G  T  L  L  G  K  A  T  K  S  S  L  N  M  T  Y  E  K  Y  N  E  D  D  K  K  W  A  K
1531  GTTAATCCAGCATTTTATTTTCCTAAAGTGACCTATACTCAGTTTACCTCTTTAGCTTCAGATAGCTTTGATCCAGGAAAAAGTGTTTCT
511   V  N  P  A  F  Y  F  P  K  V  T  Y  T  Q  F  T  S  L  A  S  D  S  F  D  P  G  K  S  V  S
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541   E  R  A  E  A  V  Y  N  F  L  T  K  L  G  Y  K  K  E  G  I  C  A  I  L  G  S  F  T  E  E
1711  TCTCAAATCAATCCTAAGCGAGCTGAAGGAGATTATTTATCTCCACCAGTTGGTGCTTCTGGAAATTCTTGGGATGATCCAGCTTGGTTG
571   S  Q  I  N  P  K  R  A  E  G  D  Y  L  S  P  P  V  G  A  S  G  N  S  W  D  D  P  A  W  L
1801  GCAATGGGTGGGTTGGATATTTACGGAAAATATCCAAACATCCTTCATAGAGGGTTGGGGTTAGGTCAATGGACAGATACATCTGATGGT
601   A  M  G  G  L  D  I  Y  G  K  Y  P  N  I  L  H  R  G  L  G  L  G  Q  W  T  D  T  S  D  G
1891  TCTGAAAGACACACTTTACTTTTAAATTATGCCAAAGCAAAAAATAAAAAATGGTACGACTTAGACCTTCAGCTTGATTTTATGCTCAAT
631   S  E  R  H  T  L  L  L  N  Y  A  K  A  K  N  K  K  W  Y  D  L  D  L  Q  L  D  F  M  L  N
1981  GGAGATACTCCAGGGAATCAAACCATGTTTAAAAATACCGCTAGTAATGCCGTCTCTTCTTCAATTCCAGAACTGACCAACTACTTCTTG
661   G  D  T  P  G  N  Q  T  M  F  K  N  T  A  S  N  A  V  S  S  S  I  P  E  L  T  N  Y  F  L
2071  ACTTACTGGGAAGGAAATCCAGGGGACAAGATTCAGGCACGTGTACAAGCCGCCCAAAATTGGTTCACTTACTTCTCAAATAATGGTGGC
691   T  Y  W  E  G  N  P  G  D  K  I  Q  A  R  V  Q  A  A  Q  N  W  F  T  Y  F  S  N  N  G  G
2161  TCTGATGCGGATATGAGCGCATCATCAAAAGAACTCTTTGAGAAATATAAAGACAAAATTAAACCTCTCCCTTCTAATAAAGAAACACAA
721   S  D  A  D  M  S  A  S  S  K  E  L  F  E  K  Y  K  D  K  I  K  P  L  P  S  N  K  E  T  Q
2251  CAAGGGCAAGGATGGCCTGGTAATGGCTATGAACCTGGGAATTGTACTTGGTATGTCTTTAATCGTCAAGCTCAAATTGGTCATAATATT
751   Q  G  Q  G  W  P  G  N  G  Y  E  P  G  N  C  T  W  Y  V  F  N  R  Q  A  Q  I  G  H  N  I
2341  AATGGCTATATGGGTAATGGTGGCCAATGGGGTTATAACTATACTAAAACTCCAGGAGCTACAATTGATTCTAAACCACAAGTGGGAGAT
781   N  G  Y  M  G  N  G  G  Q  W  G  Y  N  Y  T  K  T  P  G  A  T  I  D  S  K  P  Q  V  G  D
2431  GCGGTAAGTTTCTCTCCAGGTGTTGCAGGGTCTAGTTCAGAATATGGTCACGTGGCTCAAGTTGAAGTTGTCAATCCAGATGGTACATTT
811   A  V  S  F  S  P  G  V  A  G  S  S  S  E  Y  G  H  V  A  Q  V  E  V  V  N  P  D  G  T  F
2521  TTAGTTTCTGAAATGAACACTTTAGGCCTTTATTCAATGGGCTATCGAATGTTTAAACCAGGAGCAGGAATGACATTTGTTCACTTTAAA
841   L  V  S  E  M  N  T  L  G  L  Y  S  M  G  Y  R  M  F  K  P  G  A  G  M  T  F  V  H  F  K
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871   -      [SEQ ID NO:2]
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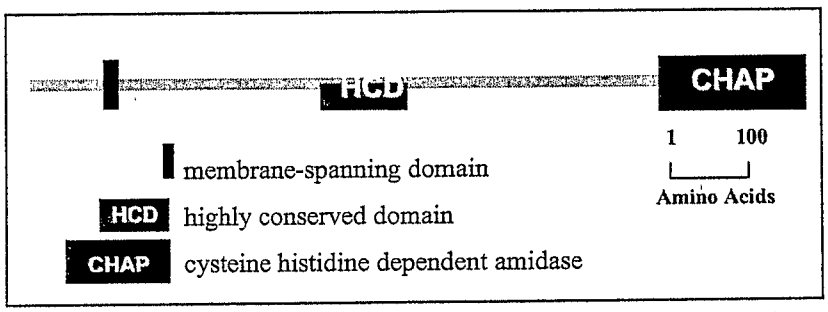
FIGURE 4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HCD
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FIGURE 5

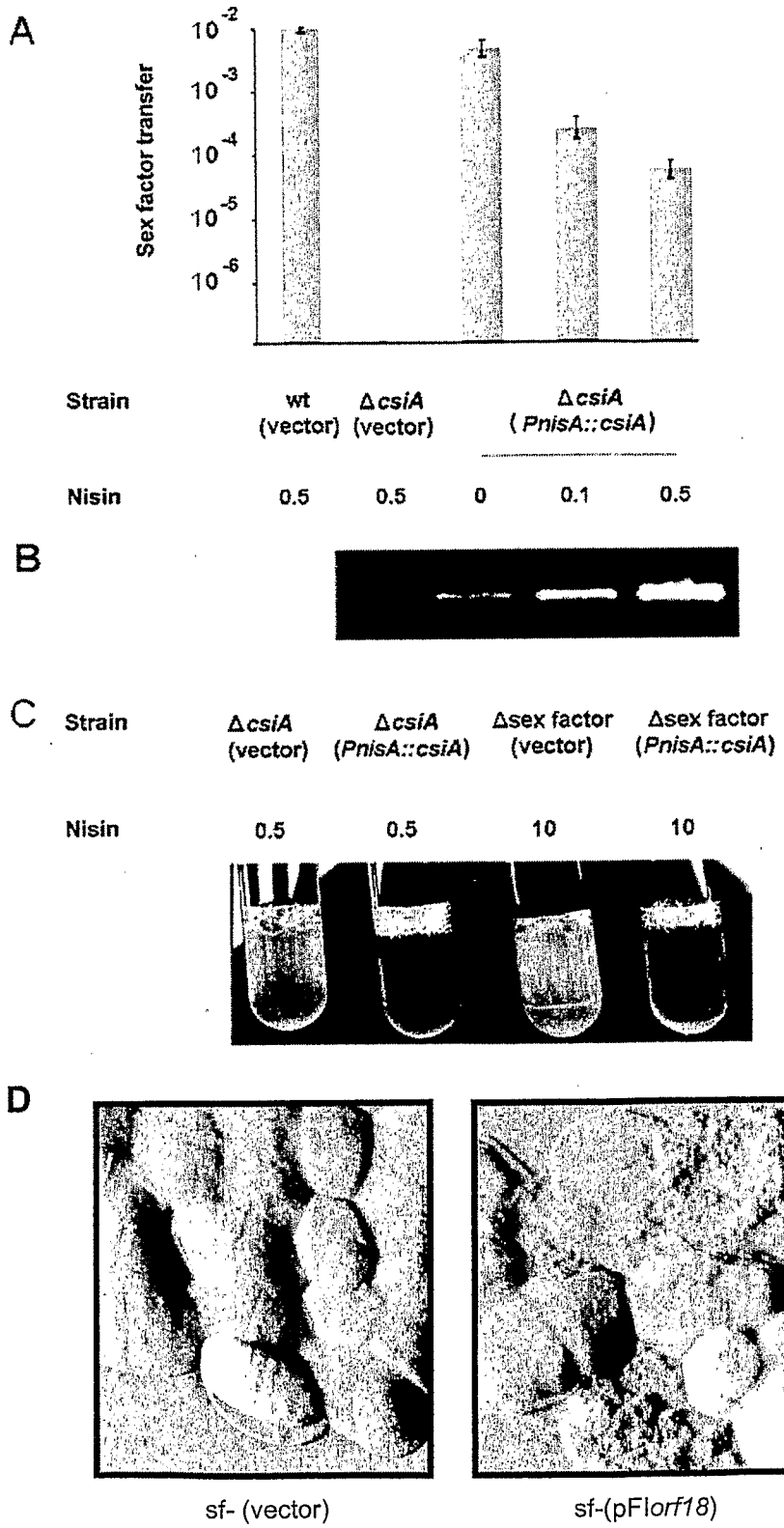
A
Sex factor transfer
10-2
10-3
10-4
10-5
10-6
Strain
wt (vector)
ΔcsiA (vector)
ΔcsiA (PnisA::csiA)
Nisin
0.5
0.5
0
0.1
0.5
B
C
Strain
ΔcsiA (vector)
ΔcsiA (PnisA::csiA)
Δsex factor (vector)
Δsex factor (PnisA::csiA)
Nisin
0.5
0.5
10
10
D
sf- (vector)
sf-(pFIorf18)

FIGURE 6

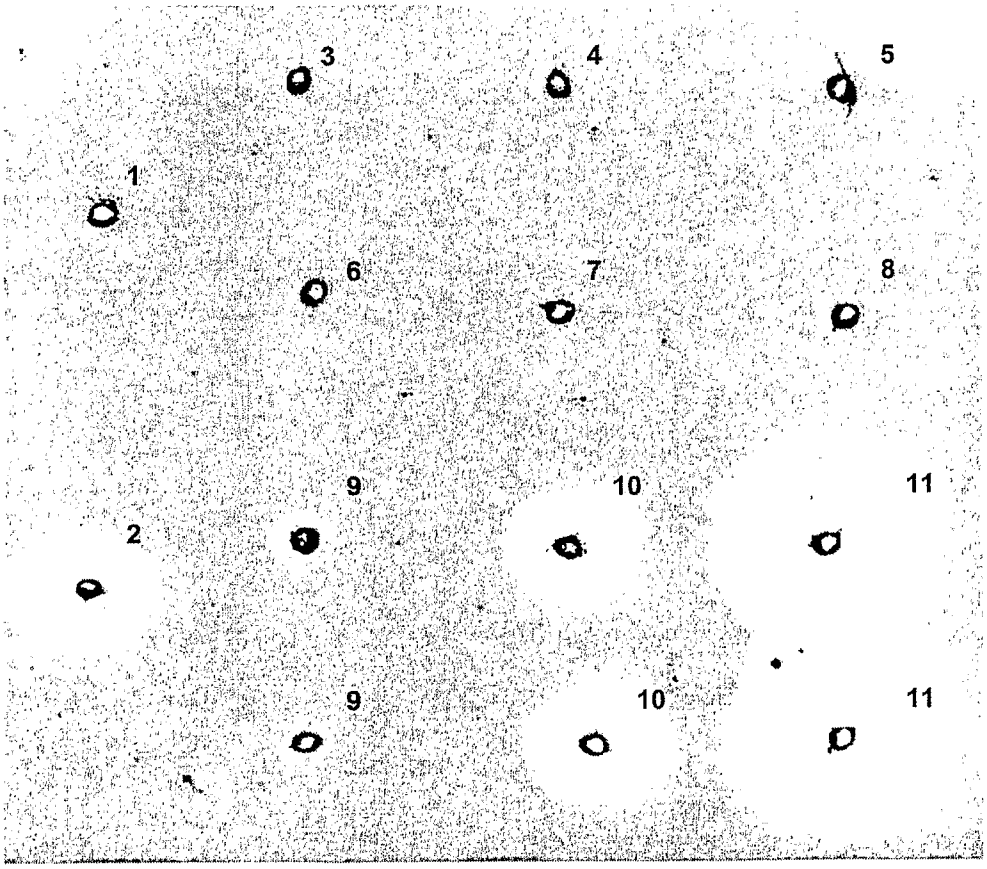

FIGURE 7

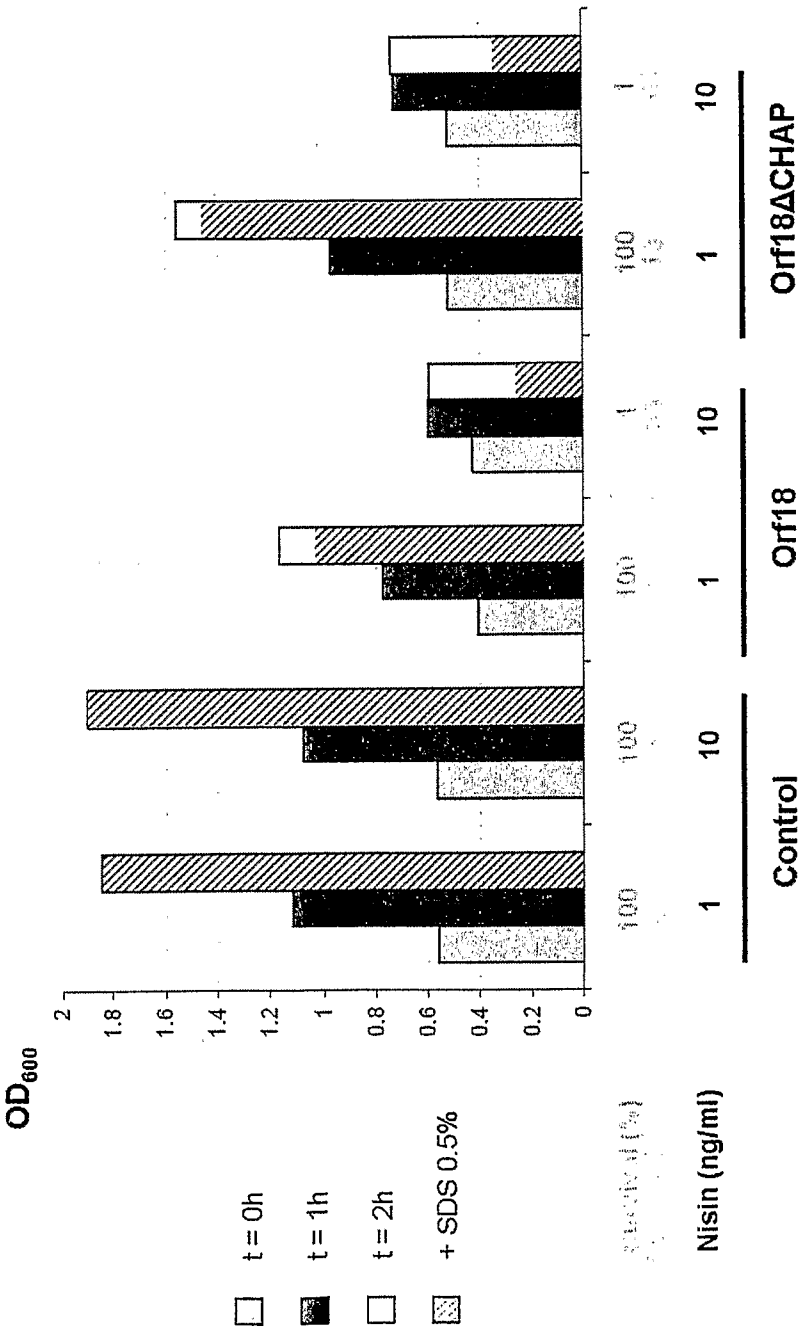
OD600
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
t = 0h
t = 1h
t = 2h
+ SDS 0.5%
Nisin (ng/ml)
1
10
100
Control
Orf18
Orf18ΔCHAP

FIGURE 8

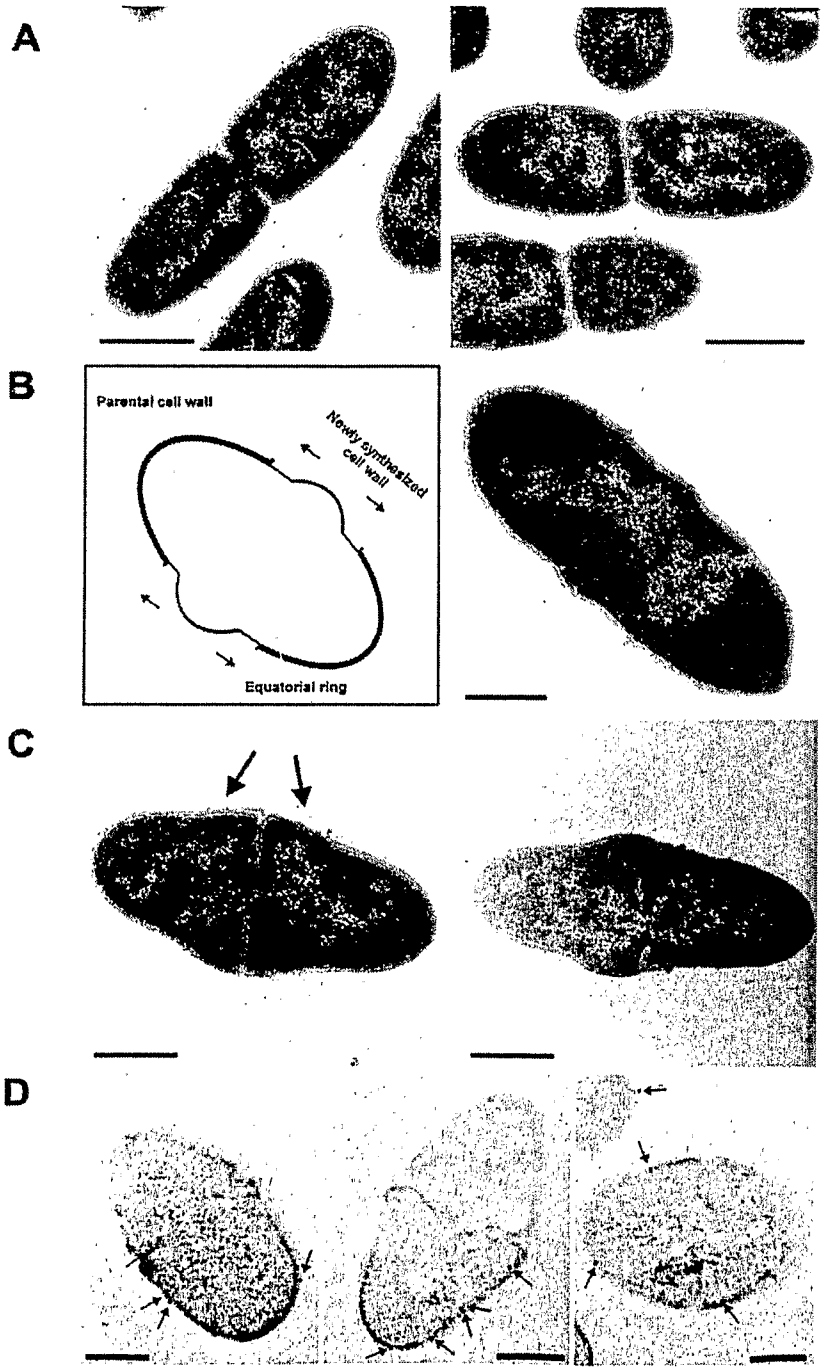
A
B
Parental cell wall
Newly synthesized cell wall
Equatorial ring
C
D

FIGURE 9

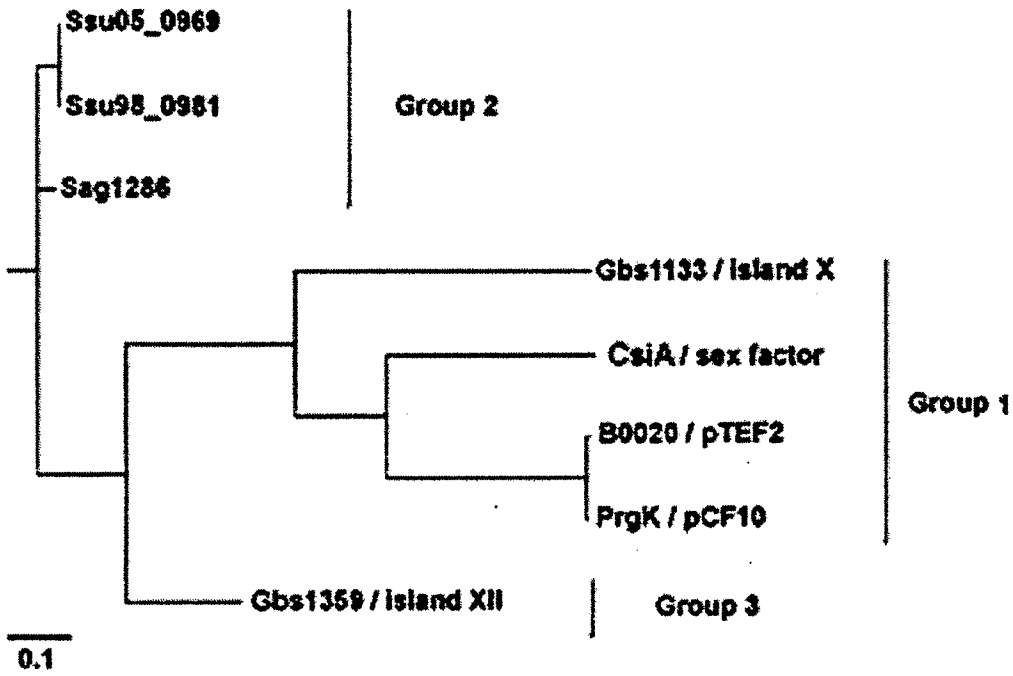
Ssu05_0969
Ssu98_0981
Sag1286
Group 2
Gbs1133 / Island X
CsiA / sex factor
B0020 / pTEF2
PrgK / pCF10
Group 1
Gbs1359 / Island XII
Group 3
0.1

FIGURE 10

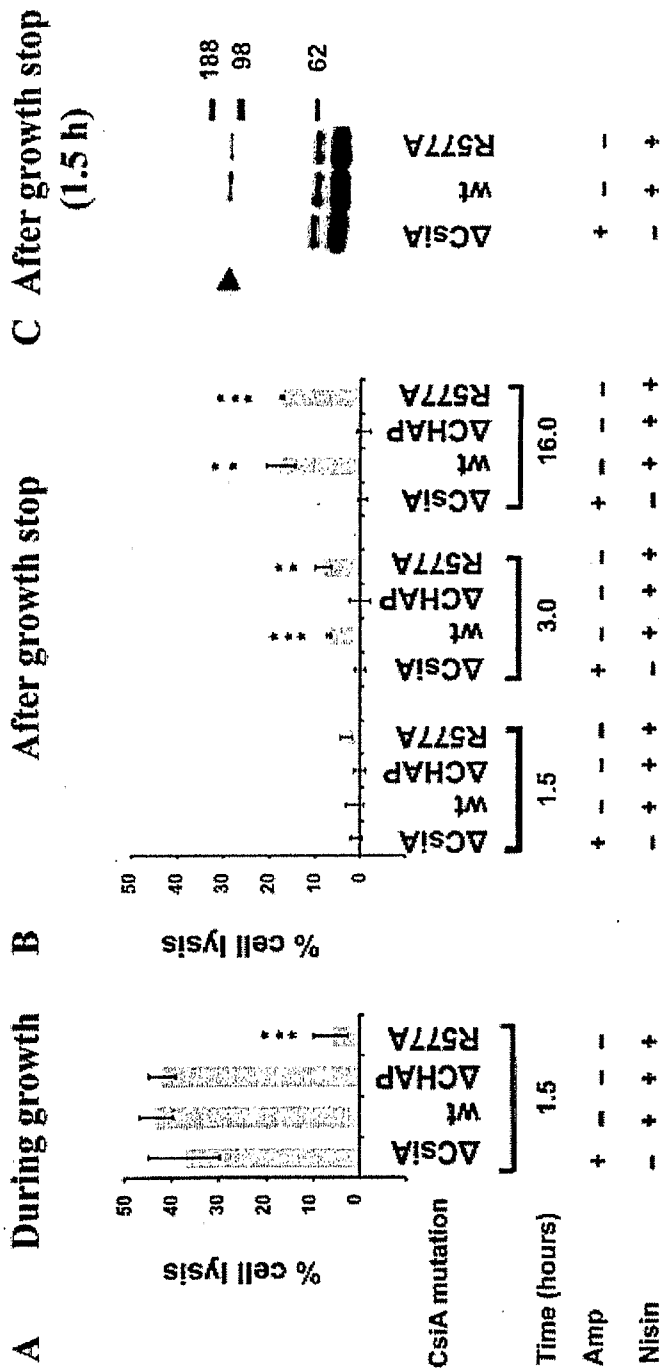
A During growth
% cell lysis
50
40
30
20
10
0
***
ΔCsiA
wt
ΔCHAP
R577A
1.5
CsiA mutation
Time (hours)
Amp
Nisin
B After growth stop
% cell lysis
50
40
30
20
10
0
**
***
**
***
ΔCsiA
wt
ΔCHAP
R577A
1.5
3.0
16.0
C After growth stop (1.5 h)
188
98
62
ΔCsiA
wt
R577A

FIGURE 11

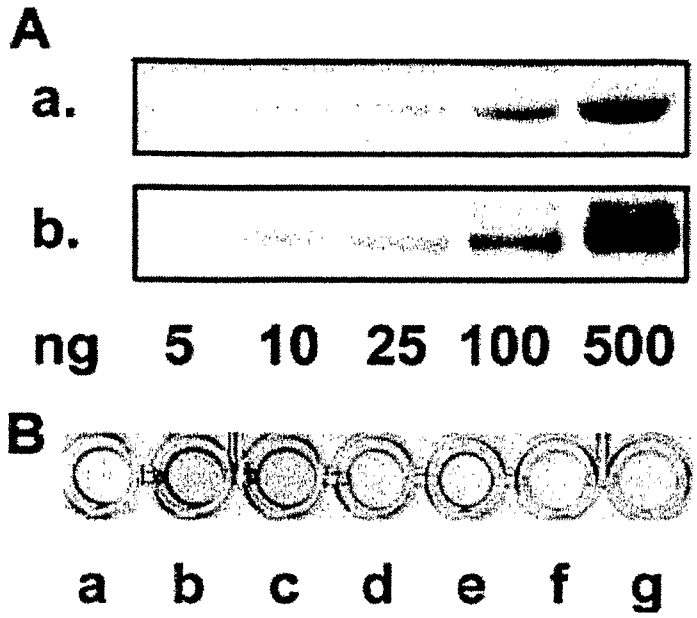

FIGURE 12