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(54) **Title**: PREPARATION OF SMALL COLONY VARIANTS OF THERAPEUTIC BACTERIA

(57) **Abstract**: The invention relates to methods of differentiation, isolation, propagation, and storage of small colony variants (SCVs) of *E. coli*, preferably *E. coli* 83972 or *E. coli* HU2117, or modified or variant forms thereof, and methods for using the prepared SCV bacteria to establish probiotic biofilms in treated subjects and/or on treated medical devices.

PREPARATION OF SMALL COLONY VARIANTS OF THERAPEUTIC BACTERIA

The present application claims priority to U.S. Provisional Application Serial No. 61/971,913, filed March 28, 2014, which is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates to methods of preparing and using small colony variants of probiotic organisms, *e.g.*, non-pathogenic strains of *E. coli.*

BACKGROUND OF THE INVENTION

Bacteriuria and pyuria are uniformly present in patients who have indwelling urinary catheters. Antimicrobial therapy may transiently eradicate the bacteria, but bacteriuria promptly recurs, and the infecting bacteria become progressively resistant to antibiotics. Antimicrobial (*e.g.*, antibiotic and/or antiseptic) treatment of asymptomatic urinary tract infections (UTI) in catheterized patients has not been shown to be of benefit, as treated and untreated catheterized patients have a similar prevalence of infection a few weeks after the end of treatment, and an equal likelihood of developing symptomatic episodes of UTI (Nicolle, L. E., Drugs Aging **22**(8): 627-39 (2005). Additionally, antimicrobial treatment of asymptomatic catheter-associated UTIs (CAUTIs) has been associated with the emergence of drug-resistant organisms, complicating management when symptomatic infections occur.

Studies have indicated that pre-colonization of the bladder with certain non-pathogenic strains of *E. coli* is a safe and effective way of preventing or reducing the *in vivo* incidence of urinary catheter colonization by a wide variety of uropathogens.

SUMMARY OF THE INVENTION

The present invention relates to the preparation and propagation of particular variant forms of non-pathogenic *E. coli.* In some embodiments, small colony variants selected for use in therapeutic preparations, *e.g.*, in freeze-dried preparations of lubricant gels containing probiotic microbes. The invention relates to methods of differentiation, isolation, propagation, and storage of small colony variants (SCVs) of *E. coli,* preferably *E. coli* 83972

or *E. coli* HU2117, or modified or variant forms thereof, and methods for using the prepared bacteria to establish biofilms in treated subjects.

In some embodiments, the technology provides methods of culturing SCVs of probiotic bacteria, preferably in liquid culture. In some embodiments, the methods comprise isolating an *E. coli* small colony variant (SCV) bacterium, inoculating a liquid growth medium with the SCV bacterium, wherein the liquid growth medium is a supplemented minimal medium comprising a a source of carbon such as a sugar or sugar alcohol, and further comprising the amino acids cysteine, methionine, serine, and lysine. Preferably, the liquid growth medium does not comprise added adenine, cytosine, guanine, uracil, yeast extract, or an enzymatic digest of complex protein. In some embodiments, the liquid culture medium comprises a buffered solution, such as a 3-(N-morpholino)propanesulfonic acid (MOPS) buffer solution. In certain preferred embodiments the MOPS solution comprises a MOPS/tricene solution.

Methods of culturing SCV bacteria further comprise incubating an inoculated liquid growth medium under conditions in which the SCV bacterial strain grows to produce a liquid culture of bacterial cells that maintain the SCV form, with minimal reversion to the normal or "large colony variant" (LCV) form. In some embodiments, the liquid culture of SCV bacterial cells comprises fewer than 50%, preferably fewer than 40%, preferably fewer than 30%, preferably fewer than 20%, preferably fewer than 10 %, preferably fewer than 5%, preferably fewer than 1%, more preferably fewer than 0.1% of corresponding normal or LCV form of the bacteria. In some preferred embodiments, the liquid culture of SCV bacterial cells is free or essentially free of corresponding LCV bacterial cells.

In some embodiments, the source of carbon comprises glycerol, and in certain preferred embodiments, the source of carbon consists essentially of glycerol, in that glycerol is the sole added carbon source in the liquid growth medium.

In some embodiments, isolating the SCV form of *E. coli* comprises isolating an SCV bacterium from urine. In some embodiments, the urine is biological, *i.e.*, it is urine produced by a human or animal, while in some embodiments, the urine is synthetic or artificial urine. In some embodiments, the SCV bacterium is cultured in urine. Culturing SCV bacteria in urine may occur in a body (*in vivo, e.g.,* in the urinary tract of an individual) or it may occur outside a body (*ex vivo*, *e.g.,* in a vessel in a laboratory).

In some embodiments, isolating an SCV bacterium comprises growing the SCV bacterium on a solid culture medium, *e.g.,* on an agar-containing medium such as MacConkey's agar or LB agar.

In some embodiments, the liquid growth medium, *e.g.,* a supplemented minimal medium as discussed above, further comprises one or more amino acids selected from asparagine, aspartic acid, glycine, phenylalanine, and tryptophan. The liquid culture medium may also contain one or more salts. In some embodiments, the liquid growth medium comprises one or more of ferrous sulfate, ammonium chloride, potassium sulfate, calcium chloride, magnesium chloride, and sodium chloride, and in some embodiments, the liquid growth medium further comprises one or more of ammonium molybdate, boric acid, cobalt chloride, cupric sulfate, manganese chloride and zinc sulfate.

In certain embodiments, the liquid growth medium is a defined medium that consists essentially of:

MOPS	40 mM
3-(N-Morpholino)-propanesulfonic acid	
Tricine	4 mM
Iron Sulfate	10 μM
Ammonium Chloride	9.5 mM
Potassium Sulfate	276 μM
Calcium Chloride Monohydrate	0.5 μM
Magnesium Chloride	525 μM
Sodium Chloride	50 mM
Ammonium Molybdate	2.92×10^{-9} M
Boric Acid	4×10^{-7} M
Cobalt Chloride	3.02×10^{-8} M
Cupric Sulfate	9.62×10^{-9} M
Manganese Chloride	8.08×10^{-8} M
Zinc Sulfate	9.74×10^{-9} M
Potassium Phosphate, Dibasic	1.32 mM
Alanine	0.798 mM

Arginine HCl	5.2 mM
Asparagine	0.4 mM
Aspartic Acid, Potassium Salt	0.4 mM
Cysteine Monohydrate HCl	0.1 mM
Glutamic Acid, Potassium Salt	0.7 mM
Glutamine	0.6 mM
Glycine	0.8 mM
Histidine monohydrate HCl	0.2 mM
Isoleucine	0.4 mM
Leucine	0.8 mM
Lysine DiHydrochloride	0.4 mM
Methionine	0.2 mM
Phenylalanine	0.4 mM
Proline	0.4 mM
Serine	10.0 mM
Threonine	0.4 mM
Tryptophane	0.1 mM
Tyrosine	0.2 mM
Valine	0.6 mM
Thiamine HCl	0.01 mM
Calcium Pantothenate	0.01 mM
ρ-aminobenzoic acid	0.01 mM
ρ-hydroxybenzoic acid	0.01 mM
2,3-dihydroxybenzoic acid	0.01 mM
Glycerol	0.4 % (w/v)
Water	

In some embodiments, the technology provides viable probiotic SCV *E. coli* bacteria in a medical lubricant gel, *e.g.,* for lubricating a medical device prior to use. In preferred embodiments, use of the probiotic gel composition induces formation of a biofilm of said *E.*

coli bacteria, *e.g.,* on the surface of a treated medical device/and or on a tissue surface exposed to the treated device, such as in the urinary tract of a patient.

In some embodiments, providing a lubricant gel containing SCV *E. coli* bacteria comprises the steps of a) providing in an aqueous fluid a mixture comprising i) a liquid culture of probiotic SCV bacterial cells, as described above, ii) a pharmaceutically acceptable gelling agent, and iii) a pharmaceutically acceptable protective agent; and b) freezing the mixture to produce a frozen preparation of bacteria mixed with lubricant gel. In some embodiments, the frozen preparation is then dried under vacuum to produce a freeze-dried preparation, *e.g.,* for stable storage.

Freeze-dried preparations of probiotic *E. coli* bacteria and lubricant gels may be prepared as described, *e.g.,* in US Patent Application No. 12/671,370, which is incorporated herein by reference in its entirety. The gelling agent is selected to provide a suitable lubricant function during use, when the preparation is either thawed (if frozen) or reconstituted with liquid (if freeze-dried). In some embodiments, the gelling agent comprises one or more of hydroxyethyl cellulose, hydroxymethyl cellulose, hydroxypropyl guar, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, sodium carboxymethyl cellulose, carbomer, alginate, gelatin, or poloxamer. In certain preferred embodiments, the gelling agent comprises or consists of hydroxyethyl cellulose.

In some embodiments, the aqueous fluid mixture containing the SCV bacteria further comprises a pharmaceutically acceptable second protective agent that is different from the first protective agent. In certain embodiments, the first and second protective agents comprise one or more of non-fat milk solids, trehalose, glycerol, betaine, sucrose, glucose, lactose, dextran, polyethylene glycol, sorbitol, mannitol, poly vinyl propylene, potassium glutamate, monosodium glutamate, Tween 20 detergent, Tween 80 detergent, and/or an amino acid hydrochloride.

In some embodiments, the technology provides a frozen or a freeze-dried composition comprising probiotic *E. coli* SCV bacterial cells, produced according to the methods described above. In some embodiments, the preparation of SCV bacterial cells comprises fewer than 50%, preferably fewer than 40%, preferably fewer than 30%, preferably fewer than 20%, preferably fewer than 10 %, preferably fewer than 5%, preferably fewer than 1%, more preferably fewer than 0.1% of corresponding normal or LCV forms of

the bacteria. In some preferred embodiments, the preparation of SCV bacterial cells is free or essentially free of corresponding LCV bacterial cells.

In some embodiments, the technology provides a method of forming a biofilm on a medical device by treating the device with a preparation of SCV *E. coli* bacterial cells. In some embodiments, the technology comprises a) providing a freeze-dried preparation comprising SCV bacterial cells, a pharmaceutically acceptable gelling agent, and a pharmaceutically acceptable protective agent; b) exposing the freeze dried preparation to an aqueous fluid to form a medically acceptable lubricant gel comprising an effective amount of the SCV bacterial cells; c) contacting the medical device with the lubricant gel to produce a treated device; and d) exposing the treated device to conditions in which a biofilm of the bacterial cells forms on the treated device and/or on a tissue exposed to the treated device.

In some embodiments, the method of forming a biofilm on a medical device comprises a) providing a frozen preparation comprising a preparation of SCV bacterial cells, *e.g.*, as described above, a pharmaceutically acceptable gelling agent, and a pharmaceutically acceptable protective agent; b) thawing the frozen preparation to form a medically acceptable lubricant gel comprising an effective amount of the SCV bacterial cells; c) contacting the medical device with the lubricant gel to produce a treated device; and d) exposing the treated device to conditions wherein a biofilm forms from the SCV bacterial cells on the treated device and/or on a tissue exposed to the treated device.

In some embodiments, the medical device is a urinary catheter. In certain embodiments, the exposing of the treated device to biofilm-forming conditions comprises contacting a subject, *e.g.*, a patient, with the treated device.

In additional embodiments, the technology provides methods of administering SCV bacterial cells to a subject, comprising a) providing a freeze-dried preparation comprising a mixture of preparation of SCV bacterial cells, a pharmaceutically acceptable gelling agent, and a pharmaceutically acceptable protective agent; b) exposing the freeze dried preparation to an aqueous fluid to form a medically acceptable lubricant gel comprising an effective amount of the SCV bacterial cells; and c) contacting the subject with the lubricant gel. In other embodiments, the technology provides methods of administering SCV bacterial cells to a subject, comprising a) providing a frozen preparation comprising a mixture of a preparation of SCV bacterial cells, a pharmaceutically acceptable gelling agent, and a pharmaceutically

acceptable protective agent; b) thawing the frozen preparation to form a medically acceptable lubricant gel comprising an effective amount of the SCV bacterial cells; and c) contacting a subject with the lubricant gel.

In certain embodiments, contacting a subject with the gel comprises contacting a medical device with the lubricant gel to produce a treated device, and contacting the subject with the treated device. In certain preferred embodiments, the medical device is a urinary catheter.

The technology further provides kits for convenient use of the compositions and methods described above. For example, in some embodiments, the technology is provided as a kit comprising, *e.g.,* i) a freeze-dried or frozen composition mixture comprising SCV *E. coli* bacterial cells, as described above. In certain embodiments in which the composition is provided in freeze-dried form, the kit may further comprise a container of sterile aqueous fluid, *e.g.*, water, for re-suspending the freeze-dried mixture to produce a lubricant gel containing an effective amount of the SCV bacteria. In some embodiments, the kit further comprises a medical device, *e.g.*, a catheter such as a urinary catheter.

DESCRIPTION OF THE DRAWING

Figures 1A-1C show the colony morphology of large and small colony variants of *E. coli* HU2117 on MacConkey agar (A), LB agar (B), or modified MOPS minimal agar (C). Large-colony variants are streaked on the left of each plate, and small-colony variants are streaked on the right.

Figure 2 shows MacConkey agar plates streaked with two different glycerol freezer stocks of SCV-form *E. coli* HU2117. The left plate shows HU2117 Stock A; the right plate shows HU2117 Stock B.

Figure 3 shows LB agar plates streaked with two different glycerol freezer stocks of SCV-form *E. coli* HU2117. The left plate shows HU2117 Stock A; the right plate shows HU2117 Stock B.

Figure 4 show MacConkey agar plates streaked with two different glycerol freezer stocks of LCV-form *E. coli* 83972. The left plate shows 83972 Stock A; the right plate shows 83972 Stock B.

Figure 5 show LB agar plates streaked with two different glycerol freezer stocks of LCV-form *E. coli* 83972. The left plate shows 83972 Stock A; the right plate shows 83972 Stock B.

Figure 6 shows MacConkey agar plates streaked with glycerol freezer stocks of SCV and LCV forms of *E. coli* 83972. The left plate shows SCV-form CON42-5; the right plate shows LCV-form CON19-4A.

Figure 7 shows LB agar plates streaked with glycerol freezer stocks of SCV and LCV forms of *E. coli* 83972. The left plate shows SCV-form CON42-5; the right plate shows LCV-form CON19-4A.

Figures 8A and 8B show MacConkey and LB agar plates streaked with glycerol stocks of SCV forms of *E. coli* 83972 and HU2117 prepared as described herein. The left plates of 8A and 8B show 83972 and the right plates show HU2117.

DEFINITIONS

To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

Small colony variant (SCV) refers to a variant of *E. coli* characterized in that, when grown on solid media, it forms colonies that are substantially smaller than the normal colony size of the corresponding parent strain. For example, SCVs of *E. coli* typically form colonies that, after overnight incubation, are pinpoint in size on the agars on which they grow, while the LCVs form colonies of normal size, *e.g.*, 1 mm or larger in diameter, when well separated from other colonies. See, for example, Fig. 1, which compares LCVs and SCVs of *E. coli* HU2117 on MacConkey's agar, Luria-Bertani (LB) agar, and on a modified MOPS minimal agar. SCV colonies of an *E.coli* strain generally measure one tenth or less the diameter of the normal or large colony variants of the same organism. While colonies are generally not formed during cultivation in liquid medium, the form of the microbe is deemed to be "SCV" if, when the liquid culture is streaked on solid culture media and incubated to form colonies, the resulting colonies are predominantly or exclusively in the SCV form.

Large colony variant as used herein refers to the variant having normal size, generally being at least 10-fold larger in diameter than SCVs of the same organism when grown identically.

As used herein, the term "subject" refers to individuals (*e.g.*, human, animal, or other organism) to be treated by the methods or compositions of the present invention. Subjects include, but are not limited to, mammals (*e.g.*, murines, simians, equines, bovines, porcines, canines, felines, and the like), and most preferably includes humans. In the context of the invention, the term "subject" generally refers to an individual who will receive or who has received treatment (*e.g.*, administration of a probiotic microbe, and optionally one or more other agents) for a condition characterized by the presence of pathogenic bacteria, or in anticipation of possible exposure to pathogenic bacteria.

The term "diagnosed," as used herein, refers to the recognition of a disease (*e.g.*, caused by the presence of pathogenic bacteria) by its signs and symptoms (*e.g.*, resistance to conventional therapies), or genetic analysis, pathological analysis, histological analysis, and the like.

As used herein the term, "*in vitro*" refers to an artificial environment and to processes or reactions that occur within an artificial environment. *In vitro* environments include, but are not limited to, test tubes and cell cultures. The term "*in vivo*" refers to the natural environment (*e.g.*, an animal or a cell) and to processes or reactions that occur within a natural environment.

As used herein, the term "virulence" refers to the degree of pathogenicity of a microorganism, *e.g.*, as indicated by the severity of the disease produced or its ability to invade the tissues of a subject. It is generally measured experimentally by the median lethal dose (LD_{50}) or median infective dose (ID_{50}). The term may also be used to refer to the competence of any infectious agent to produce pathologic effects.

As used herein, the term "effective amount" refers to the amount of a composition (*e.g.*, a probiotic microbe) sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages and is not intended to be limited to a particular formulation or administration route. It is within the skilled artisan's ability to relatively easily determine an effective amount of a probiotic microbe or other therapeutic composition.

As used herein, the term "administration" refers to the act of giving a drug, prodrug, or other agent, or therapeutic treatment (*e.g.*, compositions of the present invention) to a physiological system (*e.g.*, a subject or *in vivo*, *in vitro*, or *ex vivo* cells, tissues, and organs).

Exemplary routes of administration to the human body can be through the urethra, eyes (ophthalmic), mouth (oral), skin (transdermal), nose (nasal), lungs (inhalant), oral mucosa (buccal), ear, by injection (*e.g.*, intravenously, subcutaneously, intratumorally, intraperitoneally, *etc.*) and the like.

As used herein, the term "treating a surface" refers to the act of exposing a surface, *e.g.*, of a catheter, to one or more compositions of the present invention. Methods of treating a surface include, but are not limited to, spraying, misting, submerging, and coating.

As used herein, the term "co-administration" refers to the administration of at least two agent(s) (*e.g.*, two separate donor bacteria, each comprising a different plasmid) or therapies to a subject. In some embodiments, the co-administration of two or more agents or therapies is concurrent. In other embodiments, a first agent/therapy is administered prior to a second agent/therapy. Those of skill in the art understand that the formulations and/or routes of administration of the various agents or therapies used may vary. The appropriate dosage for co-administration can be readily determined by one skilled in the art. In some embodiments, when agents or therapies are co-administered, the respective agents or therapies are administered at lower dosages than appropriate for their administration alone. Thus, co-administration is especially desirable in embodiments where the co-administration of the agents or therapies lowers the requisite dosage of a potentially harmful (*e.g.*, toxic) agent(s).

As used herein, the term "toxic" refers to any detrimental or harmful effects on a subject, a cell (including, *e.g.*, a bacterial cell prepared according to the methods herein), or a tissue as compared to the same cell or tissue prior to the administration of the toxicant.

As used herein, the term "pharmaceutical composition" refers to the combination of an active agent (*e.g.*, a probiotic microbe) with a carrier, inert or active, making the composition especially suitable for diagnostic or therapeutic use *in vitro*, *in vivo* or *ex vivo*.

The terms "pharmaceutically acceptable" or "pharmacologically acceptable," as used herein, refer to compositions that do not substantially produce adverse reactions, *e.g.*, toxic, allergic, or immunological reactions, when administered to a subject.

As used herein, the term "topically" refers to application of the compositions of the present invention to the surface of the skin and mucosal cells and tissues (*e.g.*, epithelial,

alveolar, buccal, lingual, masticatory, or nasal mucosa, and other tissues and cells which line hollow organs or body cavities).

As used herein, the term "pharmaceutically acceptable carrier" refers to any of the standard pharmaceutical carriers including, but not limited to, phosphate buffered saline solution, water, emulsions (*e.g.*, such as an oil/water or water/oil emulsions), and various types of wetting agents, any and all solvents, dispersion media, coatings, sodium lauryl sulfate, isotonic and absorption delaying agents, disintrigrants (*e.g.*, potato starch or sodium starch glycolate), and the like. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers, and adjuvants. (See *e.g.*, Martin, Remington's Pharmaceutical Sciences, 15th Ed., Mack Publ. Co., Easton, Pa. (1975), incorporated herein by reference). Moreover, in certain embodiments, the compositions of the present invention may be formulated for horticultural or agricultural use. Such formulations include dips, sprays, seed dressings, stem injections, sprays, and mists.

As used herein, the term "pharmaceutically acceptable salt" refers to any salt (*e.g.*, obtained by reaction with an acid or a base) of a compound of the present invention that is physiologically tolerated in the target subject (*e.g.*, a mammalian subject, and/or *in vivo* or *ex vivo*, cells, tissues, or organs). "Salts" of the compounds of the present invention may be derived from inorganic or organic acids and bases. Examples of acids include, but are not limited to, hydrochloric, hydrobromic, sulfuric, nitric, perchloric, fumaric, maleic, phosphoric, glycolic, lactic, salicylic, succinic, toluene-p-sulfonic, tartaric, acetic, citric, methanesulfonic, ethanesulfonic, formic, benzoic, malonic, sulfonic, naphthalene-2-sulfonic, benzenesulfonic acid, and the like. Other acids, such as oxalic, while not in themselves pharmaceutically acceptable, may be employed in the preparation of salts useful as intermediates in obtaining the compounds of the invention and their pharmaceutically acceptable acid addition salts.

Examples of bases include, but are not limited to, alkali metal (*e.g.*, sodium) hydroxides, alkaline earth metal (*e.g.*, magnesium) hydroxides, ammonia, and compounds of formula NW_4^+, wherein W is C_{1-4} alkyl, and the like.

Examples of salts include, but are not limited to: acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate,

flucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, chloride, bromide, iodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, palmoate, pectinate, persulfate, phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, undecanoate, and the like. Other examples of salts include anions of the compounds of the present invention compounded with a suitable cation such as Na^+, NH_4^+, and NW_4^+ (wherein W is a C_{1-4} alkyl group), and the like. For therapeutic use, salts of the compounds of the present invention are contemplated as being pharmaceutically acceptable. However, salts of acids and bases that are non-pharmaceutically acceptable may also find use, for example, in the preparation or purification of a pharmaceutically acceptable compound.

For therapeutic use, salts of the compounds of the present invention are contemplated as being pharmaceutically acceptable. However, salts of acids and bases that are non-pharmaceutically acceptable may also find use, for example, in the preparation or purification of a pharmaceutically acceptable compound.

As used herein, the term "medical devices" includes any material or device that is used on, in, or through a subject's or patient's body, for example, in the course of medical treatment (*e.g.*, for a disease or injury). Medical devices include, but are not limited to, such items as medical implants, wound care devices, drug delivery devices, and body cavity and personal protection devices. The medical implants include, but are not limited to, urinary catheters, intravascular catheters, dialysis shunts, wound drain tubes, skin sutures, vascular grafts, implantable meshes, intraocular devices, heart valves, and the like. Wound care devices include, but are not limited to, general wound dressings, biologic graft materials, tape closures and dressings, and surgical incise drapes. Drug delivery devices include, but are not limited to, needles, drug delivery skin patches, drug delivery mucosal patches and medical sponges. Body cavity and personal protection devices, include, but are not limited to, tampons, sponges, surgical and examination gloves, and toothbrushes. Birth control devices include, but are not limited to, intrauterine devices (IUDs), diaphragms, and condoms.

As used herein, the term "therapeutic agent," refers to compositions that decrease the infectivity, morbidity, or onset of mortality in a subject contacted by a pathogenic microorganism or that prevent infectivity, morbidity, or onset of mortality in a host contacted by a pathogenic microorganism. As used herein, therapeutic agents encompass agents used

prophylactically, *e.g.*, in the absence of a pathogen, in view of possible future exposure to a pathogen. Such agents may additionally comprise pharmaceutically acceptable compounds (*e.g.*, adjuvants, excipients, stabilizers, diluents, and the like). In some embodiments, the therapeutic agents of the present invention are administered in the form of topical compositions, injectable compositions, ingestible compositions, and the like. When the route is topical, the form may be, for example, a solution, cream, ointment, salve or spray.

As used herein, the term "pathogen" refers to a biological agent that causes a disease state (*e.g.*, infection, cancer, *etc.*) in a host. "Pathogens" include, but are not limited to, viruses, bacteria, archaea, fungi, protozoans, mycoplasma, prions, and parasitic organisms.

As used herein, the terms "probiotic" and "probiotic microbe" are used interchangeably to refer to a live microorganism that is administered in adequate amounts to confer a health benefit on the host. See, *e.g.*, Potential Uses of Probiotics in Clinical Practice, G. Reid, *et al.*, Clinical Microbiology Reviews, Oct. 2003, p658-672, incorporated herein by reference. Probiotics are not limited to microorganisms administered by any particular route. Exemplary routes of administration to the human body can be through the eyes (ophthalmic), mouth (oral), skin (transdermal), nose (nasal), lungs (inhalant), oral mucosa (buccal), vagina, rectum, urethra, ear, by injection (*e.g.*, intravenously, subcutaneously, intratumorally, intraperitoneally, *etc.*) and the like. As used herein, the term "probiotic" includes but is not limited to naturally occurring organisms and derivatives thereof, *e.g.*, *E. coli* 83972, and *E. coli* HU2117. Probiotic organisms may also be modified, *e.g.*, through selective culturing or recombinant engineering, to have altered properties. For example, probiotic microbes configured to contain conjugatively transmissible plasmids that alter recipient cells (*e.g.*, to kill or to reduce pathogenicity of a pathogen recipient cell) also find use with the present invention. See, *e.g.*, U.S. Application Serial Numbers 11/137,950 and 11/137,948, each of which is incorporated herein by reference in its entirety.

As used herein, the term "microbe" refers to a microorganism and is intended to encompass both an individual organism, or a preparation comprising any number of the organisms.

The terms "bacteria" and "bacterium" refer to all prokaryotic organisms, including those within all of the phyla in the Kingdom Procaryotae. It is intended that the term encompass all microorganisms considered to be bacteria including *Mycoplasma, Chlamydia,*

Actinomyces, Streptomyces, and *Rickettsia*. All forms of bacteria are included within this definition including cocci, bacilli, spirochetes, spheroplasts, protoplasts, *etc.* Also included within this term are prokaryotic organisms that are Gram-negative or Gram-positive. "Gram-negative" and "Gram-positive" refer to staining patterns with the Gram-staining process, which is well known in the art. (See *e.g.*, Finegold and Martin, Diagnostic Microbiology, 6th Ed., CV Mosby St. Louis, pp. 13-15 (1982)). "Gram-positive bacteria" are bacteria that retain the primary dye used in the Gram stain, causing the stained cells to generally appear dark blue to purple under the microscope. "Gram-negative bacteria" do not retain the primary dye used in the Gram stain, but are stained by the counterstain. Thus, Gram-negative bacteria generally appear red.

As used herein, the term "microorganism" refers to any species or type of microorganism, including but not limited to, bacteria, archaea, fungi, protozoans, mycoplasma, and parasitic organisms. The present invention contemplates that a number of microorganisms encompassed therein will also be pathogenic to a subject.

As used herein, the term "fungi" is used in reference to eukaryotic organisms such as the molds and yeasts, including dimorphic fungi.

The term "non-pathogenic bacteria" or "non-pathogenic bacterium" includes all known and unknown non-pathogenic bacterium (Gram-positive or Gram-negative) and any pathogenic bacterium that has been mutated or converted to a non-pathogenic bacterium. Furthermore, a skilled artisan recognizes that some bacteria may be pathogenic to specific species and non-pathogenic to other species; thus, these bacteria can be utilized in the species in which it is non-pathogenic or mutated so that it is non-pathogenic.

As used herein, the term "non-human animals" refers to all non-human animals including, but are not limited to, vertebrates such as rodents, non-human primates, ovines, bovines, ruminants, lagomorphs, porcines, caprines, equines, canines, felines, aves, *etc.*

As used herein, the term "cell culture" refers to any *in vitro* culture of cells, including, *e.g.*, prokaryotic cells and eukaryotic cells. Included within this term are continuous cell lines (*e.g.*, with an immortal phenotype), primary cell cultures, transformed cell lines, finite cell lines (*e.g.*, non-transformed cells), bacterial cultures in or on solid or liquid media, and any other cell population maintained *in vitro*.

As used herein, the term "liquid culture" refers to a preparation of an organism that has been grown in a liquid culture medium, *e.g.*, LB broth, MOPS minimal medium, such that the resulting liquid contains a distribution of the organisms, *e.g.*, bacteria.

A "liquid culture medium" may be of any liquid composition suitable to provide nutrients to the organism to be grown. A solidifying agent, *e.g.*, agar, may be added to produce a "solid culture medium", *e.g.*, culture plates or slants, also referred to as agar plates and agar slants.

As used herein, the terms "inoculate" and "inoculation" as verbs refer to the act of introducing an organism into an environment free of that organism, *e.g.*, using a sample or colony of *E. coli* to inoculate a sterile culture medium in order to cultivate the strain of *E. coli*.

As used herein, the term "incubate" refers to holding an item or sample (*e.g.*, an inoculated culture, a chemical or enzymatic reaction mixture) at a temperature for a period of time or until a particular result (*e.g.*, orgasm growth or reaction result) occurs.

As used herein, the term "urine" refers to a liquid preparation having the essential elements found in biological urine, *i.e.*, urine produced by a human or animal. Urine may be natural or synthetic. For example, a synthetic urine preparation may comprise a mixture of Peptone l37 yeast extract, lactic acid, citric acid, sodium bicarbonate, urea uric acid, creatinine, calcium chloride, sodium chloride, iron II sulfate, magnesium sulfate, sodium sulfate, potassium dihydrogen phosphate, di-potassium hydrogen phosphate, and ammonium chloride, each at an physiological concentration in water. See, *e.g.,* Brooks and Keevil, Letters in Applied Microbiology 1997,(24):203–206, " A simple artificial urine for the growth of urinary pathogens", and Souhaila Bouatra, et al., PLOSOne, September 2013; Volume 8/ Issue 9/e73076, each of which is incorporated herein by reference in its entirety.

As used herein, the term "biofilm" refers to a cohesive matrix of organisms, *e.g.*, *E. coli* bacteria, adhered to a surface. A biofilm typically comprises an extracellular polymeric substance (comprising, *e.g.*, polysaccharides) exuded by the organism(s), which is a matrix in which the cells are embedded and which adheres the cells to each other and to the surface. A surface supporting a biofilm may be a non-biological surface (*e.g.*, the surface of a medical device) or may be biological (*e.g.*, a tissue surface in a subject). Biofilms may comprise multiple species or may be formed by a single species of microbe.

As used, the term "eukaryote" refers to organisms distinguishable from "prokaryotes." It is intended that the term encompass all organisms with cells that exhibit the usual characteristics of eukaryotes, such as the presence of a true nucleus bounded by a nuclear membrane, within which lie the chromosomes, the presence of membrane-bound organelles, and other characteristics commonly observed in eukaryotic organisms. Thus, the term includes, but is not limited to such organisms as fungi, protozoa, and animals (*e.g.*, humans).

As used herein, the term "kit" refers to any delivery system for delivering materials. In the context of reaction materials such as probiotic microbes, such delivery systems include but are not limited to systems that allow for the storage, transport, or delivery of appropriate reagents (*e.g.*, cells, buffers, culture media, selection reagents, *etc.*, in the appropriate containers) and/or devices (*e.g.*, catheters, syringes, reaction tubes or plates, culture tubes or plates) and/or supporting materials (*e.g.,* media, written instructions for performing using the materials, *etc.*) from one location to another. For example, kits include one or more enclosures (*e.g.,* boxes, bags) containing the relevant reaction reagents and/or supporting materials. As used herein, the term "fragmented kit" refers to delivery systems comprising two or more separate containers that each contains a subportion of the total kit components. The containers may be delivered to the intended recipient together or separately. For example, a first container may contain a dried composition of a microbe with a gelling agent for a particular use, while a second container contains sterile fluid such as water or buffer for dissolving or resuspending a dried composition. The term "fragmented kit" is intended to encompass kits containing Analyte Specific Reagents (ASR's) regulated under section 520(e) of the Federal Food, Drug, and Cosmetic Act, but are not limited thereto. Indeed, any delivery system comprising two or more separate containers that each contains a subportion of the total kit components are included in the term "fragmented kit." In contrast, a "combined kit" refers to a delivery system containing all of the components of a reaction materials needed for a particular use in a single container (*e.g.*, in a single box housing each of the desired components). The term "kit" includes both fragmented and combined kits.

With regards to the dried cake produced by lyophilization of a composition, the term "elegant" is used in the literature to describe a 'perfect' lyophilization product with no cracks, no shrinkage, with smooth edges, and a fluffy consistency.

As used herein, the terms "a" and "an" means at least one, and may refer to more than one.

The term "bacterial interference" as used herein refers to an antagonistic interaction between bacteria and other microorganisms to establish themselves and dominate their environment. Bacterial interference operates through several mechanisms, *e.g.*, production of antagonistic substances, changes in the bacterial microenvironment, competition for attachment sites, and reduction of needed nutritional substances.

The term "coating" as used herein refers to a layer of material covering, *e.g.*, a medical device or a portion thereof. A coating can be applied to the surface or impregnated within the material of the implant.

As used herein, the term "antimicrobial agent" refers to a composition other than a probiotic that decreases, prevents or inhibits the growth of bacterial and/or fungal organisms. Examples of antimicrobial agents include, *e.g.*, antibiotics and antiseptics.

The term "antiseptic" as used herein is defined as an antimicrobial substance that inhibits the action of microorganisms, including but not limited to alpha.-terpineol, methylisothiazolone, cetylpyridinium chloride, chloroxyleneol, hexachlorophene, chlorhexidine and other cationic biguanides, methylene chloride, iodine and iodophores, triclosan, taurinamides, nitrofurantoin, methenamine, aldehydes, azylic acid, silver, benzyl peroxide, alcohols, and carboxylic acids and salts. One skilled in the art is cognizant that these antiseptics can be used in combinations of two or more to obtain a synergistic effect. Some examples of combinations of antiseptics include a mixture of chlorhexidine, chlorhexidine and chloroxylenol, chlorhexidine and methylisothiazolone, chlorhexidine and (.alpha.-terpineol, methylisothiazolone and alpha.-terpineol; thymol and chloroxylenol; chlorhexidine and cetylpyridinium chloride; or chlorhexidine, methylisothiazolone and thymol. These combinations provide a broad spectrum of activity against a wide variety of organisms.

As used herein, the term "dried" as used in reference to a probiotic composition refers to removing the solvent component or components to levels that no longer support chemical reactions. The term is also used in reference to a composition that has been dried (*e.g.*, a dried preparation or dried composition). Those of skill in the art will appreciate that a composition may be "dried" while still having residual solvent or moisture content after lyophilization, or that a dried composition may, after the end of the drying process, absorb

moisture hygroscopically, *e.g.*, from the atmosphere. The term "dried" encompasses a composition with increased moisture content due to hygroscopic absorption.

As used herein, the term "protective agent" refers to a composition or compound that protects the activity or integrity of an active agent (*e.g.*, an enzyme, a probiotic microbe) when the active agent is exposed to certain conditions (*e.g.*, drying, freezing). In some embodiments, a protective agent protects a living organism (*e.g.*, a probiotic microbe) during a freezing process (*i.e.*, it is a "cryoprotectant"). Examples of protective agents include but are not limited to non-fat milk solids, trehalose, glycerol, betaine, sucrose, glucose, lactose, dextran, polyethylene glycol, sorbitol, mannitol, poly vinyl propylene, potassium glutamate, monosodium glutamate, Tween 20 detergent, Tween 80 detergent, and an amino acid hydrochloride.

As used herein, the term "gelling agent" refers to a composition that, when dissolved, suspended or dispersed in a fluid (*e.g.*, an aqueous fluid such as water or a buffer solution), forms a gelatinous semi-solid (*e.g.*, a lubricant gel). Examples of gelling agents include but are not limited to hydroxyethyl cellulose, hydroxymethyl cellulose, hydroxypropyl guar, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, sodium carboxymethyl cellulose, carbomer, alginate, gelatin, and poloxamer.

As used herein, the term "excipient" refers to an inactive ingredient (*i.e.*, not pharmaceutically active) added to a preparation of an active ingredient. The gelling and protective agents described herein are referred to generally as "excipients."

As used herein, the term "consists essentially of" as used in reference to a composition means that the composition consists of the recited component(s), and that the composition includes no other components that would materially change the characteristics of the recited composition (*e.g.*, does not contain other active ingredients). For example, traces of an impurity, or *de minimus* amounts of one or more additional components that do not change the characteristics of the composition would fall within the scope of the recited composition. Similarly, as used in reference to a method or series of steps, the term refers to a method of set of steps that is limited to the recited steps, admitting only *de minimus* deviation that would not materially change the characteristics of the steps or results of the recited method.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to methods and materials useful for treating surfaces with non-pathogenic organisms, *e.g.*, bacteria. In particular embodiments, the methods and materials find use in establishing a bacterial culture on a subject, *e.g.*, on the surfaces of the urinary tract of a subject, and/or on a surface of a medical device, *e.g.*, a urinary catheter. In particular embodiments, the culture comprises a biofilm. Embodiments of the invention are directed to therapeutic preparations of small colony variant (SCV) forms of bacteria, *e.g.*, *E. coli*, for use in establishing a culture and/or a biofilm in the urinary tract of a subject.

Embodiments of the invention are described in this Description, and in the Summary of the Invention, above, which is incorporated here by reference. Although the invention has been described in connection with specific embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. For example, discussions herein pertaining to *E. coli* 83972 also encompass *E. coli* HU2117, as HU2117 is a version of 83972 engineered to have a deletion in the *papG* gene. The growth characteristics of these strains have been observed to be the same.

Small colony variants (SCVs) are a naturally occurring subpopulation of bacteria that forms colonies on solid media that are markedly smaller than the colonies formed by the parental bacteria. SCVs, *e.g.*, of *E. coli,* may be about one tenth of the diameter of the normal or "large colony variant" (LCV) form of the same strain grown on the same solid medium. See, *e.g.*, Proctor, et al., Nature Rev. Micro., 4:295-305 (2006). During development of the technology, it has been observed that SCV forms of *E. coli* 83972 and *E. coli* HU2117 are particularly useful in establishing therapeutic biofilms, *e.g.*, on catheter surfaces and/or in the urinary tract of a treated subject.

Isolates of non-pathogenic *E. coli* 83972 and/or *E. coli* HU2117 from subjects having asymptomatic urinary tract infections show a mixture of small and large colony forms of these strains. As discussed in more detail below, the small and large colony variants can be readily distinguished by looking at colony morphology on agar plates, with MacConkey's agar being particularly revealing of the size difference.

During development of the present technology, it was determined that standard rich media typically used for broth culture of bacteria (*e.g.*, Luria Bertani (LB) or other media supplemented with a complete source of amino acids, such as with yeast extract, tryptone,

peptone, *etc.*) promotes reversion from the SCV form to the LCV form in *E. coli* 83972 and *E. coli* HU2117.

In addition, the SCV forms of these *E. coli* strains are auxotrophic for numerous amino acids, while the LCV forms of the same strains are not. Thus, the SCV strains require supplemented media for growth and minimal media can be used to select *against* their growth. For example, the SCVs of *E. coli* 83972 and *E. coli* HU2117 have absolute requirements for the amino acids cysteine, methionine, serine, and lysine, and less essential requirements for asparagine, aspartic acid, glycine, phenylalanine, and tryptophan. In contrast, the LCV forms are not auxotrophs and may readily be grown in MOPS minimal medium supplemented with a carbon source (*e.g.*, 0.2% of either glucose or glycerol). Thus, it can be seen that it is relatively simple to find growth conditions to *reduce* the occurrence of SCVs, and difficult to find growth conditions that select *for* growth of the SCV forms and against the LCV forms. This makes it difficult to produce liquid cultures in which the population of cells is predominantly in SCV form, and still more difficult to produce liquid cultures that are essentially completely SCV-form bacteria.

The present invention relates to the development of methods for isolation, growth, and storage of SCVs of *E. coli* strains, particularly non-pathogenic strains *E. coli* 83972, *E. coli* HU2117, and variants thereof or derived therefrom.

The present invention is directed to methods and compositions for the production of cultures that are predominantly SCV, preferably completely SCV, such that any LCV-form *E. coli* in the liquid culture are reduced in number or are non-existent. Thus, an aspect of the invention is the identification of growth conditions that identify and maintain the SCV form of *E. coli* strains, *e.g.*, *E. coli* 83972 and *E. coli* HU2117, for use in manufacturing probiotic preparations, *e.g.*, for coating catheters. In preferred embodiments, the bacteria are grown in liquid culture without the use of antimicrobial components, *e.g.*, copper (Hirsch, J Bacteriol. 81:448-58 (1961); 2-methyl-1,4-naphthoquinone (see, *e.g.*, Colwell, J Bacteriol. 52(4):417-22 (1946).

An aspect of the present invention is selection of an SCV form of *E. coli* for therapeutic use, *e.g.*, to create biofilms. It has been determined that using the SCV form of probiotic *E. coli* in the compositions and methods of the technology described herein is effective in producing a biofilm of the probiotic strains in the urinary tract of the treated

subject and/or on the surface of a urinary catheter. The technology thus provides methods of differentiation, isolation, propagation, and storage of small colony variants (SCVs) of *E. coli,* preferably *E. coli* 83972 or *E. coli* HU2117, or modified or variant forms thereof, and methods for using the prepared bacteria to establish biofilms in treated subjects.

The technology further provides methods and compositions for delivering an effective amount of probiotic SCV *E. coli* to a subject or patient. While not limiting the present invention to any particular formulation or mode of administration, in some preferred embodiments, the probiotic microbe is present in prepared lubricant gel mixture in a concentration of about 10^7 to cfu 10^9 per ml of lubricant gel.

Growth characteristics of *E. coli* strains 83972 and HU2117

During development of the technology and cultivation of the *E. coli* strains discussed herein, *E. coli* strains 83972 and HU2117, observation of small and large colony variant growth characteristics of these strains indicated the following:

1. Streaking a mixed culture comprising SCV and LCV forms of the strain on MacConkey's agar showed the two colony morphologies clearly and distinctly, such that fresh SCV colonies could be selected for further steps. The difference in morphology, although apparent on LB agar, is less clear on this medium.

2. Extended culture of *E. coli* HU2117 in rich media (*e.g.*, LB or tryptic soy) results in a shift of the culture from small to large colony morphology. While small colony variant populations can give rise to cells having the large colony phenotype under several culture conditions tested, it is difficult to shift the phenotype of a particular LCV isolate to produce a population of SCV microbes.

3. Passage of SCVs isolates on MacConkey's agar maintains the small colony phenotype over time.

4. Small colony variants did not grow on MOPS minimal medium or in MOPS minimal broth.

5. MOPS minimal medium inoculated with an SCV inoculum will after several days, become turbid, predominantly by the growth of cells having LCV morphology, indicating that the development of turbidity occurs either because of conversion of cells from small- to large-colony morphology, and/or from the survival and replication of a minority

population of LCVs in the SCV inoculum. This indicates that using only minimal medium for culture of these strains would favor cultivation of LCV forms of *E. coli* HU2117 and 83972.

6. During development of the technology, it was determined that use of glycerol as the carbon source (in place of glucose, for example) reduces the rate of reversion to LCV form, and thereby helps to maintain the SCV morphology during liquid culturing.

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure that follows, the following abbreviations apply: ºC. (degrees Celsius); cm (centimeters); g (grams); l or L (liters); ml or mL (milliliters); µl or µL (microliters); µg (micrograms); µm (micrometers); µM (micromolar); µmol (micromoles); mg (milligrams); mm (millimeters); mM (millimolar); mmol (millimoles); M (molar); mol (moles); ng (nanograms); nm (nanometers); nmol (nanomoles); N (normal); pmol (picomoles); bp (base pairs); cfu (colony forming units).

EXAMPLE 1

Defining conditions for culturing small colony variant forms of *E. coli* 83972 and HU2117

The initial characterization of *E. coli* 83972 and HU2117 started by trying to define a good synthetic growth medium for the culture of these *E. coli* strains. Strain HU2117 is an engineered variant of 83972 having an engineered deletion in the *papG* gene.

Several different media (both agar plates and broths) were tried to find an acceptable media that could be used for manufacturing, and that could also aid in distinguishing between the small and large colony variants. Two commercially available mediums, EZ Rich Defined medium and MOPS minimal medium, were purchased from Teknova (Hollister, CA). Two different carbon sources, glucose and glycerol, were used in combination with these two

different media. The strains were also streaked and maintained on different agars, including MacConkey's, TSA and LB agars. The media and agars were formulated as shown below:

MOPS Defined Medium (*e.g.*, EZ Rich Defined Medium)

Component #	Description	Amount
1	10X MOPS Mixture	100 mL
2	0.132 M K_2HP04	10 mL
3	10X ACGU	100 mL
4	5X Supplement EZ	200 mL
	Sterile H_2O*	580 mL
	20% Glucose or Glycerol	10 mL
	Total	1000 mL

MOPS Minimal Medium

Component #	Description	Amount
1	10X MOPS Mixture (see below)	100 mL
2	0.132 M K_2HP04	10 mL
	Sterile H_2O*	880 mL
	20% Glucose or Glycerol	10 mL
	Total	1000 mL

MOPS Media Components

#1 MOPS Modified Rich Buffer	10X Concentration	1X Concntration
MOPS (MW 209.3)	400mM	40mM
Tricine (MW 179.2)	40mM	4.0mM
Iron Sulfate Stock	0.1mM	0.01mM
Ammonium Chloride	95mM	9.5mM
Potassium Sulfate	2.76mM	0.276mM
Calcium Chloride	0.005mM	0.0005mM
Magnesium Chloride	5.25mM	0.525mM
Sodium Chloride	500mM	50mM
Ammonium Molybdate	$3x10^{-8}$ M	$3x10^{\wedge-9}$ M
Boric Acid	$4x10^{-6}$ M	$4x10^{-7}$ M
Cobalt Chloride	$3x10^{-7}$ M	$3x10^{-8}$ M
Cupric Sulfate	10^{-7} M	10^{-8} M
Manganese Chloride	$8x10^{-7}$ M	$8x10^{-8}$ M
Zinc Sulfate	10^{-7} M	10^{-8} M

#2 Potassium Phosphate Dibsasic Solution	100X Concentration	1X Concntration
Potassium Phosphate Dibasic	132mM	1.32mM

#3 ACGU Solution	10X concentration	1X concntration
Potassium Hydroxide	15mM	1.5mM
Adenine	2.0mM	0.2mM
Cytosine	2.0mM	0.2mM
Uracil	2.0mM	0.2mM
Guanine	2.0mM	0.2mM

#4 5X Supplement	5X concentration	1X concntration
L-Alanine	4.0mM	0.8mM
L-Arginine	26mM	5.2mM
L-Asparigine	2.0mM	0.4mM
L-Aspartic Acid, Potassium Salt	2.0mM	0.4mM
L-Glutamic Acid, Potassium Salt	3.3mM	0.66mM
L-Glutamine	3.0mM	0.6mM
L-Glycine	4.0mM	0.8mM
L-Histidine HCl H2O	1.0mM	0.2mM
L-Isoleucine	2.0mM	0.4mM
L-Proline	2.0mM	0.4mM
L-Serine	50mM	10mM
L-Threonine	2.0mM	0.4mM
L-Tryptophan	0.5mM	0.1mM
L-Valine	3.0mM	0.6mM
L-Leucine	4.0mM	0.8mM
L-Lysine	2.0mM	0.4mM
L-Methionine	1.0mM	0.2mM
L-Phenylalinine	2.0mM	0.4mM
L-Cysteine HCl	0.5mM	0.1mM
L-Tyrosine	1.0mM	0.2mM
Thiamine	0.05mM	0.01mM
Calcium Pantothenate	0.05mM	0.01mM
para-Amino Benzoic Acid	0.05mM	0.01mM
para-Hydroxy benzoic Acid	0.05mM	0.01mM
di Hydroxy Benzoic Acid	0.05mM	0.01mM

20% Glucose Solution	10X concentration	1X concentration
Glucose	20%	2.00%
20% Glycerol Solution	**10X concentration**	**1X concentration**
Glycerol	20%	2.00%

(See, *e.g.*, Teknova; F. C. Neidhardt, P. L. Bloch, and D. F. Smith. 1974. Culture medium for enterobacteria. J Bacteriol 119(3): 736-747)

MacConkey's Agar:

Peptone (Difco) or Gelysate (BBL) 17.0 g
Proteose peptone (Difco) or Polypeptone (BBL) 3.0 g
Lactose 10.0 g
NaCl 5.0 g
Crystal Violet 1.0 mg
Neutral Red 30.0 mg
Bile Salts 1.5 g
Agar 13.5 g
Distilled Water Add to make 1 Liter

pH adjusted to 7.1 +/- 0.2

Tryptic Soy Agar

Casein peptone (pancreatic) 15.0 g
Soya peptone (papainic) 5.0 g
Sodium chloride 5.0 g
Agar 15.0 g
Distilled water added to make 1 Liter
pH adjusted to 7.3 +/- 0.2.

Luria-Bertani broth and agar:

Tryptone 10 g
Yeast Extract 5 g
NaCl 10 g
Agar 15.0 g
Distilled water added to make 1 Liter

Broth forms of the media above omit the agar component. All are sterilized, *e.g.*, by autoclaving at 15 psi, from 121-124°C for about 15 minutes.

i. Comparison of small and large colony variants of *E. coli* HU2117 on three agars

During the course of developing the technology, it was observed that the SCV forms of these *E. coli* strains will not grow in modified MOPS minimal broth or agar, formulated as described above. The appearances of large and small colony variants on MacConkey's agar, LB agar, and modified MOPS minimal agar are shown in Figure 1. The left side of each plate was streaked with LCV form, and the right side of each plate was streaked with SCV form. All plates were incubated for the same period of time. While the colony size difference can be observed on the LB plate (Fig. 1B), the streaks are similar enough in appearance that the SCV form could be overlooked. In contrast, on the MacConkey's plate (Fig. 1A), the SCV colonies have grow to a small fraction of the size of the LCV colonies. The MOPs minimal media plate clearly shows that the SCV form do not grow on this medium.

ii. Small-to-large colony conversion rate in rich media.

Using glycerol stocks of strains having exclusively SCVs, it was possible to calculate small-to-large colony conversion rates in different types of liquid media.

EZ Rich defined glycerol medium

SCV stocks were used to inoculate a broth of EZ Rich defined glycerol medium (described above). After incubation, aliquots of the cultures were diluted and plated onto LB agar (to show all colonies) and onto MOPS minimal glucose agar plates (on which SCVs cannot grow) so that the relative amounts of each morphological type in the culture could be determined. Only two colonies grew on the MOPS minimal glucose plates, showing that the broth culture (and the original stock) to be nearly completely composed of SCV isolates.

Calculating the total number of large colonies that would be in this culture (accounting for dilutions) and the total number of *E. coli* HU2117 in the culture, the per bacterium conversion rate in glycerol-based medium was determined to be 1.4×10^{-9}.

EZ Rich defined glucose medium

Similar stocks were used to inoculate EZ Rich defined glucose medium. After incubation, aliquots of the cultures were diluted and plated onto LB agar and onto MOPS

minimal glucose agar plates, so that the relative amounts of each morphological type in the liquid culture could be determined. Thirty three colonies grew on the MOPS minimal glucose plates, showing a per bacterium conversion rate in the glucose-based medium of $3.04x10^{-8}$.

These data show that using glycerol as a sole carbon source for in liquid cultures produces a slower rate of conversion, and that glycerol is preferred over glucose for maintenance of the SCV morphology.

In a specific embodiment, the liquid culture medium for preparing SCV forms of *E. coli*, *e.g.*, *strains* 83972 and HU2117 have the following formulation (prior to inoculation):

MOPS 3-(N-Morpholino)- propanesulfonic acid	40 mM
Tricine	4 mM
Iron Sulfate	10 μM
Ammonium Chloride	9.5 mM
Potassium Sulfate	276 μM
Calcium Chloride Monohydrate	0.5 μM
Magnesium Chloride	525 μM
Sodium Chloride	50 mM
Ammonium Molybdate	$2.92x10^{-9}$ M
Boric Acid	$4x10^{-7}$ M
Cobalt Chloride	$3.02x10^{-8}$ M
Cupric Sulfate	$9.62x10^{-9}$ M
Manganese Chloride	$8.08x10^{-8}$ M
Zinc Sulfate	$9.74x10^{-9}$ M
Potassium Phosphate, Dibasic	1.32 mM
Alanine	0.798 mM
Arginine HCl	5.2 mM
Asparagine	0.4 mM
Aspartic Acid, Potassium Salt	0.4 mM
Cysteine Monohydrate HCl	0.1 mM
Glutamic Acid, Potassium Salt	0.7 mM
Glutamine	0.6 mM
Glycine	0.8 mM
Histidine monohydrate HCl	0.2 mM

Isoleucine	0.4 mM
Leucine	0.8 mM
Lysine DiHydrochloride	0.4 mM
Methionine	0.2 mM
Phenylalanine	0.4 mM
Proline	0.4 mM
Serine	10.0 mM
Threonine	0.4 mM
Tryptophane	0.1 mM
Tyrosine	0.2 mM
Valine	0.6 mM
Thiamine HCl	0.01 mM
Calcium Pantothenate	0.01 mM
ρ-aminobenzoic acid	0.01 mM
ρ-hydroxybenzoic acid	0.01 mM
2,3-dihydroxybenzoic acid	0.01 mM
Glycerol	0.4 % (w/v)
Water	

Table 1

iii. Stored isolates of SCVs maintain SCV conformation

Two different glycerol stocks of the SCV form of *E. coli* HU2117 (stocks A and B, see Figs. 2 and 3) and two different stocks of the LCV form of *E. coli* 83972 (stocks A and B, see Figs. 4 and 5) were used to streak both LB agar and MacConkey agar plates.

After 24 hours of incubation at 37°C, good growth was observed on all plates that were streaked. Figure 2 and Figure 3, show resulting MacConkey agar plates and LB agar plates that were streaked with *E. coli* HU2117. All colonies on both the MacConkey agar plates (Fig. 2) and on the LB plates (Fig 3) showed a small colony morphology.

The plates streaked with the stocks of *E. coli* 83972 almost uniformly showed the LCV derivative. Both stocks A and B of *E. coli* 83972 looked identical when these were streaked out onto either MacConkey agar (Fig. 4) or LB agar (Fig. 5). It is noted that these plates were left in the 37°C for the same amount of time as the HU2117 streaked plates

shown in Figs. 2 and 3, which demonstrates that the difference in colony size is not a result of different incubation times.

To prepare additional stocks of SCV cells, *e.g.*, *E. coli* HU2117 SCV cells, for storage, *e.g.*, at -80°C, approximately 50 colonies are inoculated into a 1-liter flask and containing 125 mL of the modified EZ rich defined medium described in Table 1, above. The cells are grown for 16 hours, and all the culture in the flask is harvested by centrifugation. The pellet is resuspended in about 11 mL of modified EZ rich defined medium. When the cells are resuspended, 11 mL of 2X freezing medium (the same modified EZ rich defined medium containing 50% glycerol) is added and the cells are placed on ice. The cell suspension is chilled on ice for 60 minutes before aliquoting into vials, *e.g.*, at a volume of 1.0 mL/vial (3.6×10^9 cfu/mL). After the cells are aliquoted into vials, they are frozen and stored at -80°C. Preferably, vials that are removed from -80°C storage are used only once.

iv. Comparison of small and large colony variants of *E. coli* 83972

One MacConkey agar plate and one LB agar plate were streaked with each of an SCV-form of *E. coli* 83972 ("CON42-5") and an LCV-form of *E. coli* 83972 ("CON19-4A"). The plates were incubated for 24 hours at 37°C, and are shown in Figs. 6 and 7.

The colony size variants on the MacConkey agar plates shown in Fig. 6 are readily distinguishable from each other. The plate streaked with CON42-5 (on the left) shows the small colony morphology, largely pinpoint colonies, and the plates streaked with the LCV-form CON19-4A isolate (on the right) clearly show large colony morphology. The same colony morphologies are observed on the LB agar plates streaked with the same glycerol freezer stocks (Fig. 7).

v. Confirmation that small and large colony variants are the same strain

SCV and LCV forms of *E. coli* HU2117, having the different growth requirements discussed above, were characterized to verify that they were genetically identical. To isolate large-colony variants, *E. coli* HU2117was streaked directly onto modified MOPS minimal medium, which only supports the growth of large-colony variants, and on MacConkey agar, which supports the growth of both large- and small-colony variants. After 40 hours of growth at 37°C, several large colonies were obtained on the MOPS minimal agar plates. Both large-

and small-colony variants were streaked onto MacConkey agar, then restreaked onto MacConkey agar and Luria-Bertani (LB) agars, and incubated at 37°C for 18 hours. Representative comparisons showing large- and small-colony variants on both MacConkey agar and LB agar are shown in Figure 1. Analysis of both colony variants shows the serotype of both variants to be O6:H1, which is the same for both strain HU2117 and the wild-type strain 83972.

vi. Confirmation of strain identity

The identity is further confirmed by PCR amplification. Both 83972 and HU2117 possess a 1.6kb cryptic plasmid that is unique to these strains, the presence of which distinguishes these strains from other *E. coli* strains. In addition, the *papG* gene of HU2117 has an engineered 803bp deletion that easily distinguishes HU2117 from its parental strain 83972, and from other *E. coli* strains that possess the *pap* operon. PCR using primer pairs specific for cryptic plasmid and for the *papG* deletion confirms that both SCV and LCV isolates are *E. coli* HU2117.

By way of example, Table 2 describes a panel of tests that may be used to verify the identity of HU2117 strain:

Tests for:	Method	Specification (Result)
papG minus genotype	PCR and sequencing	PCR amplification of a strain-specific 1584 bp fragment The flanking region of the deleted *papG* should not show any unexpected alteration compared to that of *E. coli* 83972
Genetic verification of species	Phylogenetic analysis of 16S rRNA sequence	Phylogenetically closest to *Escherichia coli*
Biochemical verification of species	β-glucuronidase activity	Blue-color colony on Chromocult TBX agar medium (EMD Biosciences)
Plasmid ID	PCR	Amplification of three fragments specific to the plasmid
RFLP	PFGE	Unique patterns of RFLP specific to *E. coli* HU2117, distinguishable from other *E. coli* strains
Antibiotic susceptibility	Growth in the presence of antibiotics	Susceptible to all antibiotics tested

Table 2

EXAMPLE 2

Preparation of a freeze-dried lubricant gel containing SCV-form *E. coli* HU2117

This example provides an exemplary method of producing a freeze-dried lubricant gel containing an effective amount of SCV-form of *E. coli* HU2117. Additional freeze-dried preparations and methods of making and using them are described, *e.g.*, in US Patent Publication 2009/0041727, published February 12, 2009, which is incorporated herein in its entirety, for all purposes.

By way of example and not by way of limitation, the starting quantity is selected so as to maintain an effective level of viability in cells freeze-dried in a composition comprising a gelling agent. For example, in some embodiments, a preferred concentration of viable SCV cells might be approximately 10^8 cfu/ml. If a vial (or other container) of the preparation is to be suspended or dissolved, *e.g.*, in 10 ml of water, the dried cake in the vial would optimally have approximately 10^9 viable cells.

Cell preparation

One 2 liter flask of cells is grown from 1ml of SCV HU2117 seed stock inoculated into 1L Modified EZ Rich Defined Glycerol medium, incubated at 37±1°C for 8 hrs with constant shaking at 250 RPM, or to an OD_{600} of about 2-2.3.

The cells are collected by centrifugation, *e.g.*, at 4°C, at 6000 RPM for 8 min. The pelleted cells are washed, *e.g.*, twice with 0.9% saline and once with 10 mM citrate buffer, pH 7.0.

The pelleted cells are resuspended into 2-3 ml of buffer, *e.g.*, of 10 mM citrate buffer, pH 7.0, for a final volume of approximately 10 ml.

The concentration of the resuspended cells may be determined using plate counts.

Lyophilization

0.5 ml of resuspended cells are mixed with 1.5 ml of an excipient, e.g, 5 to 10% sucrose, and a sterile lubricant gel, *e.g.*, 10 ml of 2% autoclaved hydroxyethyl cellulose (HEC).

The mixture is lyophilized, *e.g.*, as described below"

Process step	Step description
Loading	Incubate at 5°C and one atmosphere for 60 min
Freezing	Ramp shelf to -45°C at an average controlled rate of 5°C/min. Control shelf at target set point of -45°C for 285 min.
Primary drying/ Secondary drying	Evacuate chamber, control at a target set point of 60 mTorr. (a) Ramp shelf to -30°C at an average controlled rate of 0.2°C/min. Control shelf at target set point of -30°C for 2850 min. (b) Ramp shelf to -22°C at an average controlled rate of 0.2°C/min. Control shelf at target set point of -22°C for 1080 min. (c) Ramp shelf to -10°C at an average controlled rate of

	0.2°C/min. Control shelf at target set point of -10°C for 600 min. (d) Control chamber pressure at a target set point of 60 mTorr. Ramp shelf to 25 °C at controlled average rate of 0.2°C/min Control shelf at target set point for 720 min

After drying, the dried cake may be resuspended, *e.g.,* in about 10 to 12 ml of distilled water, for testing to determine bacterial viability and/or for use as a lubricant gel, *e.g.*, to coat a catheter prior to insertion, wherein the catheter is lubricated and the subject into whom the catheter is inserted are inoculated in a manner conducive to formation of a biofilm of *E. coli* HU2117 in the urinary tract and/or on the inserted catheter.

Using the growth conditions described herein above, the proportion of large colonies in a final product can be maintained at an extremely low level (*e.g.*, frequency of 1 in $1.0x10^8$ cfu/ml).

All publications and patents mentioned in the above specification are herein incorporated by reference for all purposes. Various modifications and variations of the described compositions and methods of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the relevant fields are intended to be within the scope of the present invention.

CLAIMS

We Claim:

1. A method of culturing probiotic bacteria, comprising:

a) isolating an *E. coli* small colony variant (SCV) bacterium;

b) inoculating a liquid growth medium with said SCV bacterium, wherein said liquid growth medium is a supplemented minimal medium comprising:

i) a buffer solution;

ii) a sugar or sugar alcohol; and

iii) cysteine, methionine, serine, and lysine,

wherein said liquid growth medium does not comprise added adenine, cytosine, guanine, uracil, yeast extract, or an enzymatic digest of complex protein;

c) incubating said liquid growth medium under conditions wherein said SCV bacterial strain grows to produce a liquid culture of SCV bacterial cells.

2. The method of claim 1, wherein said sugar or sugar alcohol comprises glycerol.

3. The method of claim 2, wherein said sugar or sugar alcohol consists of glycerol as the sole added carbon source in said liquid growth medium.

4. The method of claim 1, wherein said *E. coli* SCV bacterium is selected from *E. coli* 83972 and *E. coli* HU2117, or a variant or derivative thereof.

5. The method of claim 1, wherein in said liquid culture of SCV bacterial cells comprises fewer than 50%, preferably fewer than 40%, preferably fewer than 30%, preferably fewer than 20%, preferably fewer than 10 %, preferably fewer than 5%, preferably

fewer than 1%, more preferably fewer than 0.1% of corresponding large colony variant (LCV) bacterial cells.

6. The method of claim 1, wherein said liquid culture of SCV bacterial cells is free of corresponding LCV bacterial cells.

7. The method of claim 1 wherein said isolating comprises isolating an SCV bacterium from urine.

8. The method of claim 1, wherein said isolating comprises growing an SCV bacterium in urine, wherein said urine comprises one or more of natural urine and synthetic urine.

9. The method of claim 1, wherein said isolating comprises growing said SCV bacterium on a solid culture medium.

10. The method of claim 9, wherein said solid culture medium is MacConkey's agar.

11. The method of claim 1, wherein said buffer solution is a 3-(N-morpholino)propanesulfonic acid (MOPS) buffer solution.

12. The method of claim 11, wherein said MOPS buffer is MOPS/tricine.

13. The method of claim 1, wherein said liquid growth medium further comprises one or more amino acids selected from asparagine, aspartic acid, glycine, phenylalanine, and tryptophan.

14. The method of claim 1, wherein said liquid growth medium comprises one or more of ferrous sulfate, ammonium chloride, potassium sulfate, calcium chloride, magnesium chloride, and sodium chloride.

15. The method of claim 1, wherein said liquid growth medium further comprises one or more of ammonium molybdate, boric acid, cobalt chloride, cupric sulfate, manganese chloride and zinc sulfate.

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16. The method of claim 1, wherein said liquid growth medium consists essentially of:

MOPS (3-(N-morpholino)-propanesulfonic acid)	40 mM
Tricine	4 mM
Iron Sulfate	10 μM
Ammonium Chloride	9.5 mM
Potassium Sulfate	276 μM
Calcium Chloride Monohydrate	0.5 μM
Magnesium Chloride	525 μM
Sodium Chloride	50 mM
Ammonium Molybdate	2.92×10^{-9} M
Boric Acid	4×10^{-7} M
Cobalt Chloride	3.02×10^{-8} M
Cupric Sulfate	9.62×10^{-9} M
Manganese Chloride	8.08×10^{-8} M
Zinc Sulfate	9.74×10^{-9} M
Potassium Phosphate, Dibasic	1.32 mM
Alanine	0.798 mM
Arginine HCl	5.2 mM
Asparagine	0.4 mM
Aspartic Acid, Potassium Salt	0.4 mM
Cysteine Monohydrate HCl	0.1 mM
Glutamic Acid, Potassium Salt	0.7 mM
Glutamine	0.6 mM

Glycine	0.8 mM
Histidine monohydrate HCl	0.2 mM
Isoleucine	0.4 mM
Leucine	0.8 mM
Lysine DiHydrochloride	0.4 mM
Methionine	0.2 mM
Phenylalanine	0.4 mM
Proline	0.4 mM
Serine	10.0 mM
Threonine	0.4 mM
Tryptophane	0.1 mM
Tyrosine	0.2 mM
Valine	0.6 mM
Thiamine HCl	0.01 mM
Calcium Pantothenate	0.01 mM
ρ-aminobenzoic acid	0.01 mM
ρ-hydroxybenzoic acid	0.01 mM
2,3-dihydroxybenzoic acid	0.01 mM
Glycerol	0.4 % (w/v)
Water	

17. A method of preparing a medical lubricant gel comprising probiotic SCV bacterial cells, comprising:

a) providing in an aqueous fluid a mixture comprising:

i) a liquid culture of probiotic SCV bacterial cells;

ii) a pharmaceutically acceptable gelling agent; and

iii) a pharmaceutically acceptable first protective agent; and

b) freezing said mixture to produce a frozen preparation.

18. The method of claim 17, wherein said frozen preparation is dried under vacuum to produce a dried preparation.

19. The method of claim 17 or claim 18, wherein said liquid culture of probiotic SCV bacterial cells is a liquid culture of *E. coli* SCV bacterial cells produced according to any one of claims 1-16.

20. The method of claim 17, wherein said gelling agent is selected from the group consisting of hydroxyethyl cellulose, hydroxymethyl cellulose, hydroxypropyl guar, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, sodium carboxymethyl cellulose, carbomer, alginate, gelatin, and poloxamer.

21. The method of claim 17, wherein said mixture further comprises a pharmaceutically acceptable second protective agent, wherein said second protective agent is different from said first protective agent, said first and said second protective agents are selected from group consisting of non-fat milk solids, trehalose, glycerol, betaine, sucrose, glucose, lactose, dextran, polyethylene glycol, sorbitol, mannitol, poly vinyl propylene, potassium glutamate, monosodium glutamate, Tween 20 detergent, Tween 80 detergent, and an amino acid hydrochloride.

22. A frozen composition comprising probiotic SCV bacterial cells produced according to the method of claim 17.

23. A freeze-dried composition for preparing a medical lubricant gel comprising probiotic SCV bacterial cells, said composition produced by the method of claim 18.

24. The composition of claim 22 or claim 23, produced according to a method of claim 19

25. The composition of claim 24, wherein said *E. coli* SCV bacterial cells are selected from *E. coli* 83972 and *E. coli* HU2117 cells, or a variant or derivative thereof.

26. The composition of claim 22 or claim 23, wherein in said preparation of SCV bacterial cells comprises fewer than 50%, preferably fewer than 40%, preferably fewer than 30%, preferably fewer than 20%, preferably fewer than 10 %, preferably fewer than 5%, preferably fewer than 1%, more preferably fewer than 0.1% of corresponding LCV bacterial cells.

27. The composition of claim 22 or claim 23, wherein said preparation of SCV bacterial cells is free of corresponding LCV bacterial cells.

28. The composition of claim 22 or claim 23, wherein said gelling agent is selected from the group consisting of hydroxyethyl cellulose, hydroxymethyl cellulose, hydroxypropyl guar, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, sodium carboxymethyl cellulose, carbomer, alginate, gelatin, and poloxamer.

29. The composition of claim 22 or claim 23, wherein said first protective agent is selected from the group consisting of non-fat milk solids, sucrose, trehalose, glycerol, betaine, glucose, lactose, dextran, polyethylene glycol, sorbitol, mannitol, poly vinyl propylene, potassium glutamate, monosodium glutamate, Tween 20 detergent, Tween 80 detergent, and an amino acid hydrochloride.

30. The composition of claim 22 or claim 23, further comprising a pharmaceutically acceptable second protective agent, wherein said second protective agent is different from said first protective agent, and is selected from the group consisting of non-fat milk solids, trehalose, glycerol, betaine, sucrose, glucose, lactose, dextran, polyethylene glycol, sorbitol, mannitol, poly vinyl propylene, potassium glutamate, monosodium glutamate, Tween 20 detergent, Tween 80 detergent, and an amino acid hydrochloride.

31. A method of forming a biofilm on a medical device, comprising:

a) providing a freeze-dried preparation comprising SCV bacterial cells, a pharmaceutically acceptable gelling agent, and a pharmaceutically acceptable protective agent;

b) exposing said freeze dried preparation to an aqueous fluid to form a medically acceptable lubricant gel comprising an effective amount of said SCV bacterial cells;

c) contacting said medical device with said lubricant gel to produce a treated device; and

d) exposing said treated device to conditions wherein a biofilm of said SCV bacterial cells forms on said treated device.

32. A method of forming a biofilm on a medical device, comprising:

a) providing a frozen preparation comprising a preparation of SCV bacterial cells, a pharmaceutically acceptable gelling agent, and a pharmaceutically acceptable protective agent;

b) thawing said frozen preparation to form a medically acceptable lubricant gel comprising an effective amount of said SCV bacterial cells;

c) contacting said medical device with said lubricant gel to produce a treated device; and

d) exposing said treated device to conditions wherein a biofilm of said SCV bacterial cells forms on said treated device.

33. The method of claim 31 or claim 32, wherein said medical device is a urinary catheter.

34. The method of claim 33, wherein said exposing of said treated device comprises contacting a subject with said treated device.

35. A method of administering SCV bacterial cells to a subject, comprising:

a) providing a freeze-dried preparation comprising a preparation of SCV bacterial cells, a pharmaceutically acceptable gelling agent, and a pharmaceutically acceptable protective agent;

b) exposing said freeze dried preparation to an aqueous fluid to form a medically acceptable lubricant gel comprising an effective amount of said SCV bacterial cells; and

c) contacting said subject with said lubricant gel.

36. A method of administering SCV bacterial cells to a subject, comprising:

a) providing a frozen preparation comprising a preparation of SCV bacterial cells, a pharmaceutically acceptable gelling agent, and a pharmaceutically acceptable protective agent;

b) thawing said frozen preparation to form a medically acceptable lubricant gel comprising an effective amount of said SCV bacterial cells; and

c) contacting said subject with said lubricant gel.

37. The method of claim 35 or claim 36, wherein contacting said subject with said gel comprises contacting a medical device with said lubricant gel to produce a treated device, and contacting said subject with said treated device.

38. The method of claim 37, wherein said medical device is a urinary catheter.

39. The method of claim 35 or claim 36, wherein said SCV bacterial cells are *E. coli* HU2117 or *E. coli* 83972.

40. A kit comprising a composition according to any one of claims 22-30.

41. The kit of claim 40, further comprising a container of sterile aqueous fluid.

42. The kit of claim 41, wherein said sterile aqueous fluid is water.

43. The kit of claim 40, further comprising a medical device.

44. The kit of claim 42, wherein said medical device comprises a urinary catheter.

45. The method of any one of claims 1-3, wherein said *E. coli* SCV bacterium is selected from *E. coli* 83972 and *E. coli* HU2117, or a variant or derivative thereof.

46. The method of any one of claims 1-3 and 45, wherein in said liquid culture of SCV bacterial cells comprises fewer than 50%, preferably fewer than 40%, preferably fewer than 30%, preferably fewer than 20%, preferably fewer than 10 %, preferably fewer than 5%, preferably fewer than 1%, more preferably fewer than 0.1% of corresponding LCV bacterial cells.

47. The method of claim 46, wherein said liquid culture of SCV bacterial cells is free of corresponding LCV bacterial cells.

48. The method of any one of claims 1-3 and 45-47, wherein said isolating comprises isolating an SCV bacterium from urine.

49. The method of any one of claims 1-3 and 45-48, wherein said isolating comprises growing an SCV bacterium in urine, wherein said urine comprises one or more of natural urine and synthetic urine.

50. The method of any one of claims 1-3 and 45-49, wherein said isolating comprises growing said SCV bacterium on a solid culture medium.

51. The method of claim 50, wherein said solid culture medium is MacConkey's agar.

52. The method of any one of claims 1-3 and 45-51, wherein said buffer solution is a 3-(N-morpholino)propanesulfonic acid (MOPS) buffer solution.

53. The method of claim 52, wherein said MOPS buffer is MOPS/tricine.

54. The method of any one of claims 1-3 and 45-53, wherein said liquid growth medium further comprises one or more amino acids selected from asparagine, aspartic acid, glycine, phenylalanine, and tryptophan.

55. The method of any one of claims 1-3 and 45-54, wherein said liquid growth medium comprises one or more of ferrous sulfate, ammonium chloride, potassium sulfate, calcium chloride, magnesium chloride, and sodium chloride.

56. The method of any one of claims 1-3 and 45-55, wherein said liquid growth medium further comprises one or more of ammonium molybdate, boric acid, cobalt chloride, cupric sulfate, manganese chloride and zinc sulfate.

57. The method of any one of claims 1-3 and 45-56, wherein said liquid growth medium consists essentially of:

MOPS (3-(N-morpholino)-propanesulfonic acid)	40 mM
Tricine	4 mM
Iron Sulfate	10 μM
Ammonium Chloride	9.5 mM
Potassium Sulfate	276 μM
Calcium Chloride Monohydrate	0.5 μM
Magnesium Chloride	525 μM
Sodium Chloride	50 mM
Ammonium Molybdate	2.92×10^{-9} M
Boric Acid	4×10^{-7} M
Cobalt Chloride	3.02×10^{-8} M
Cupric Sulfate	9.62×10^{-9} M

Manganese Chloride	$8.08x10^{-8}$ M
Zinc Sulfate	$9.74x10^{-9}$ M
Potassium Phosphate, Dibasic	1.32 mM
Alanine	0.798 mM
Arginine HCl	5.2 mM
Asparagine	0.4 mM
Aspartic Acid, Potassium Salt	0.4 mM
Cysteine Monohydrate HCl	0.1 mM
Glutamic Acid, Potassium Salt	0.7 mM
Glutamine	0.6 mM
Glycine	0.8 mM
Histidine monohydrate HCl	0.2 mM
Isoleucine	0.4 mM
Leucine	0.8 mM
Lysine DiHydrochloride	0.4 mM
Methionine	0.2 mM
Phenylalanine	0.4 mM
Proline	0.4 mM
Serine	10.0 mM
Threonine	0.4 mM
Tryptophane	0.1 mM
Tyrosine	0.2 mM
Valine	0.6 mM
Thiamine HCl	0.01 mM
Calcium Pantothenate	0.01 mM
ρ-aminobenzoic acid	0.01 mM
ρ-hydroxybenzoic acid	0.01 mM
2,3-dihydroxybenzoic acid	0.01 mM
Glycerol	0.4 % (w/v)
Water	

58. A method of preparing a medical lubricant gel comprising probiotic SCV bacterial cells, comprising:

a) providing in an aqueous fluid a mixture comprising:

i) a liquid culture of E. coli SCV bacterial cells produced according to any one of claims 1-3 and 45-57;

ii) a pharmaceutically acceptable gelling agent; and

iii) a pharmaceutically acceptable first protective agent; and

b) freezing said mixture to produce a frozen preparation.

59. The method of claim 58, wherein said frozen preparation is dried under vacuum to produce a dried preparation.

60. The method of claim 58 or claim 59, wherein said gelling agent is selected from the group consisting of hydroxyethyl cellulose, hydroxymethyl cellulose, hydroxypropyl guar, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, sodium carboxymethyl cellulose, carbomer, alginate, gelatin, and poloxamer.

61. The method of any one of claims 58-60, wherein said mixture further comprises a pharmaceutically acceptable second protective agent, wherein said second protective agent is different from said first protective agent, said first and said second protective agents are selected from group consisting of non-fat milk solids, trehalose, glycerol, betaine, sucrose, glucose, lactose, dextran, polyethylene glycol, sorbitol, mannitol, poly vinyl propylene, potassium glutamate, monosodium glutamate, Tween 20 detergent, Tween 80 detergent, and an amino acid hydrochloride.

62. A frozen composition comprising probiotic SCV bacterial cells produced according to the method of any one of claims 58 and 60-61.

63. A freeze-dried composition for preparing a medical lubricant gel comprising probiotic SCV bacterial cells, said composition produced by the method of any one of claims 59-61.

64. A kit comprising a composition according to claim 62 and/or claim 63.

65. The kit of claim 64, further comprising a container of sterile aqueous fluid, preferably sterile water.

66. The kit according to any one of claims 64-65, further comprising a medical device.

67. The kit of claim 66, wherein said medical device comprises a urinary catheter.

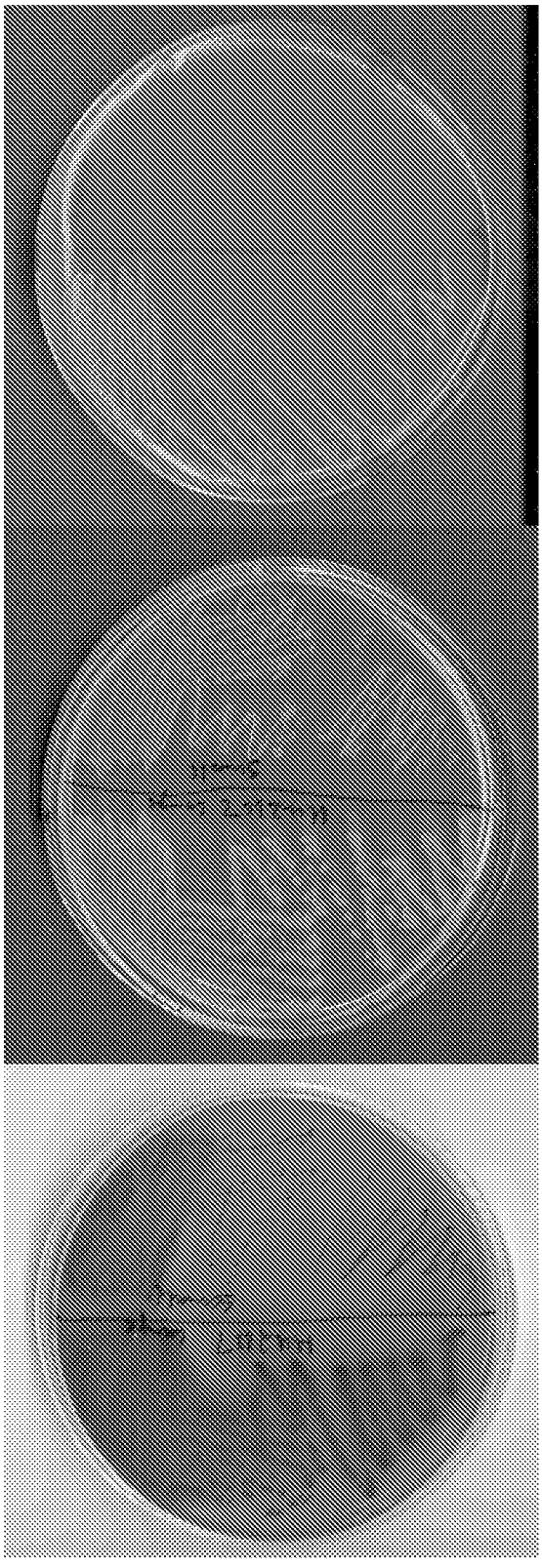


FIG. 1C

FIG. 1B

FIG. 1A

FIG. 2

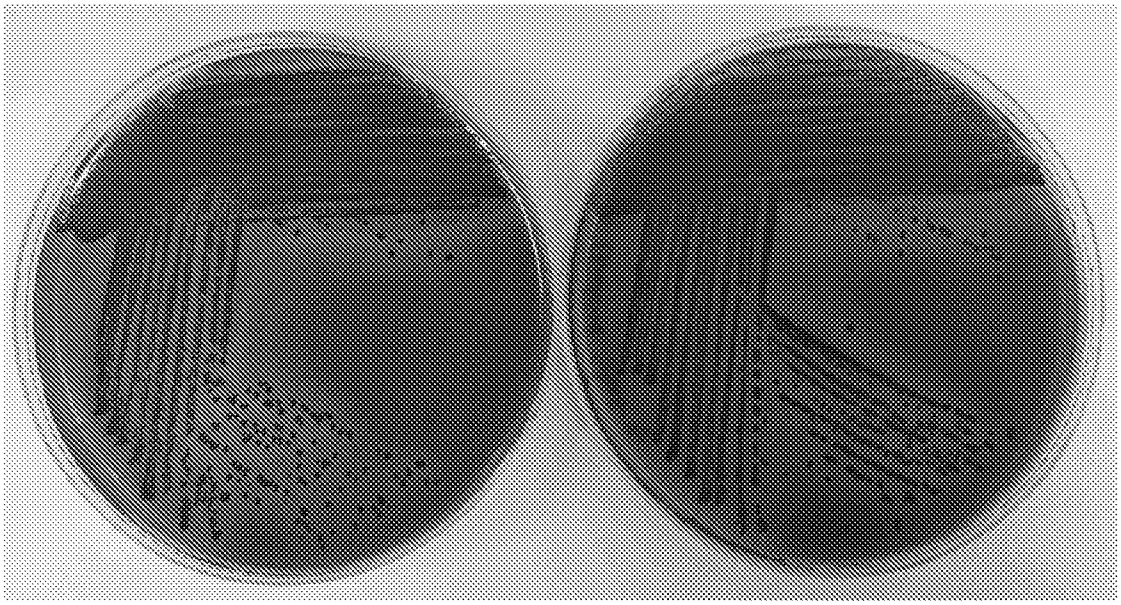


FIG. 3

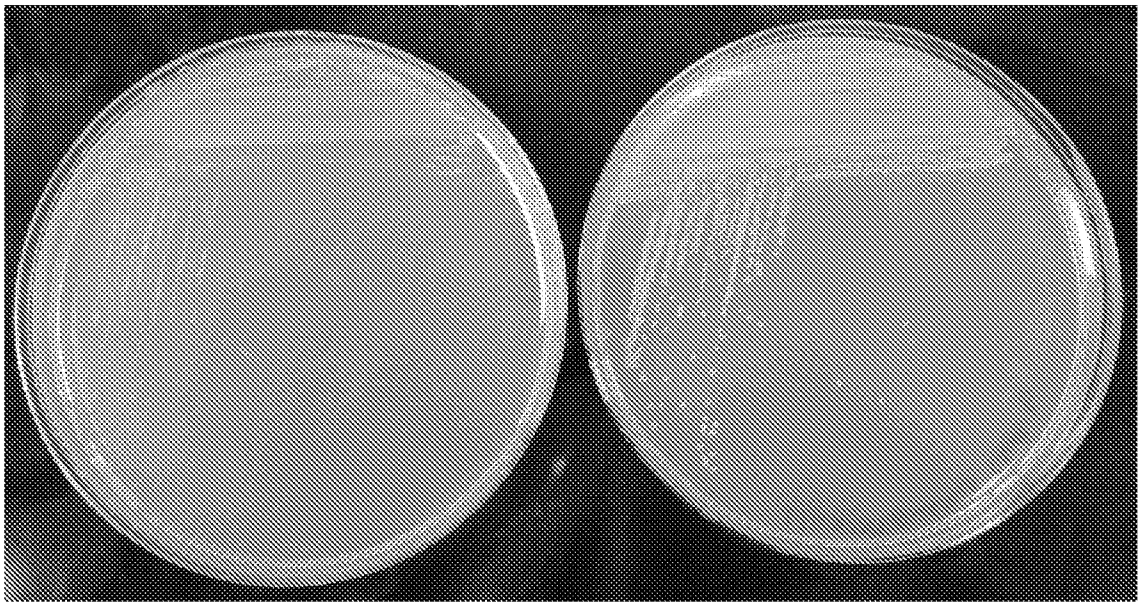


FIG. 4

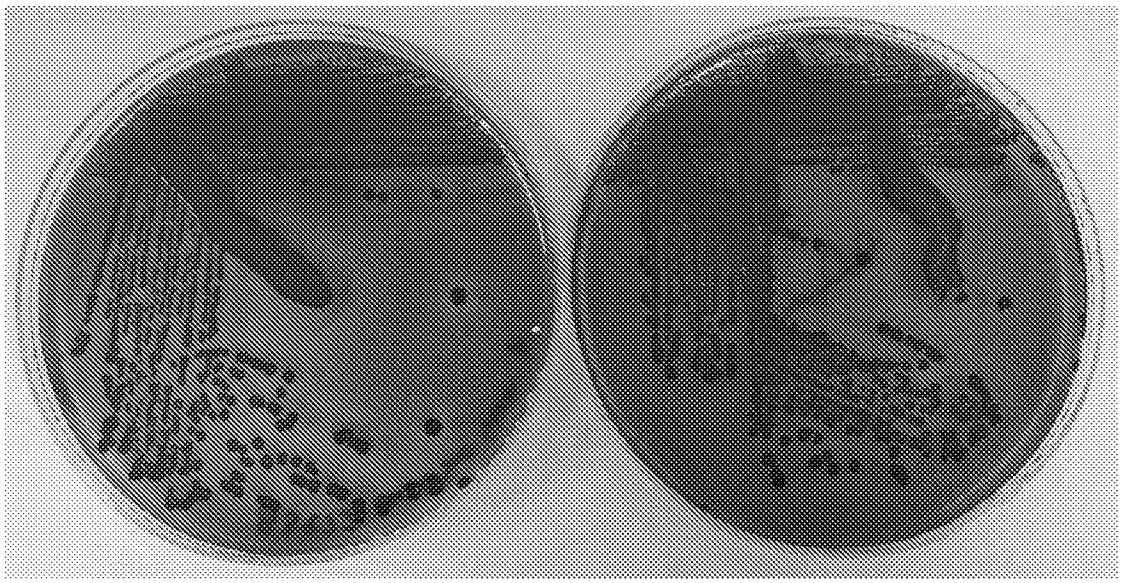


FIG. 5

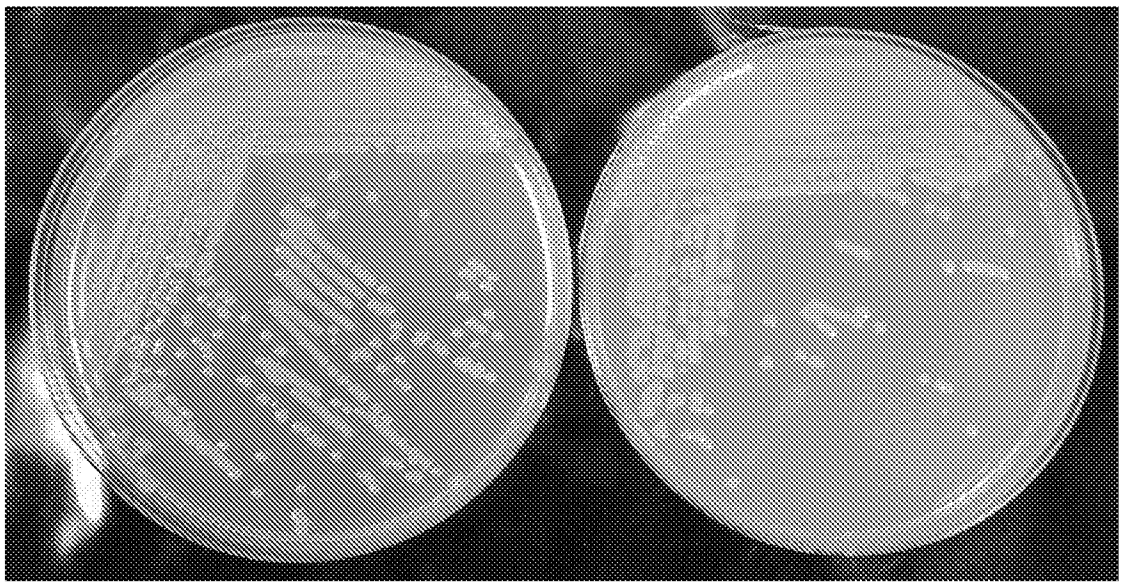


FIG. 6

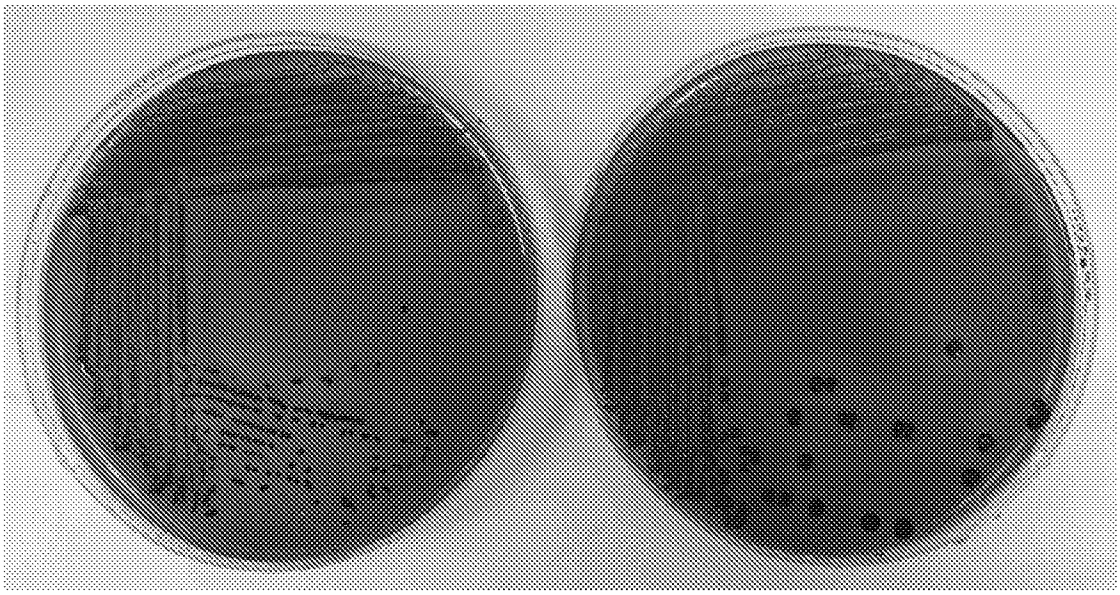


FIG. 7

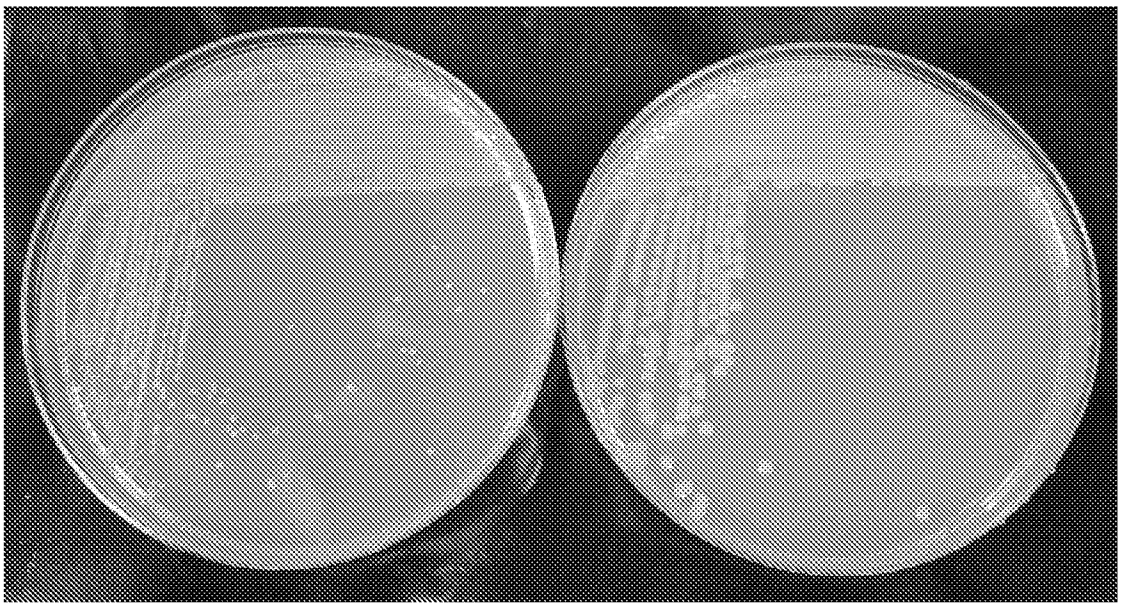


FIG. 8A

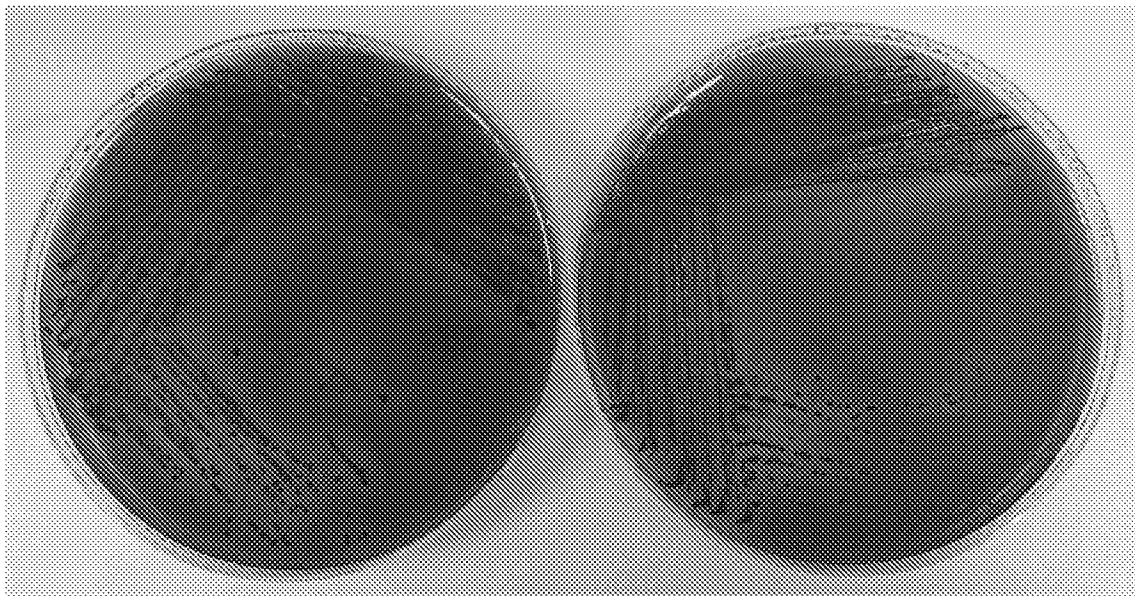
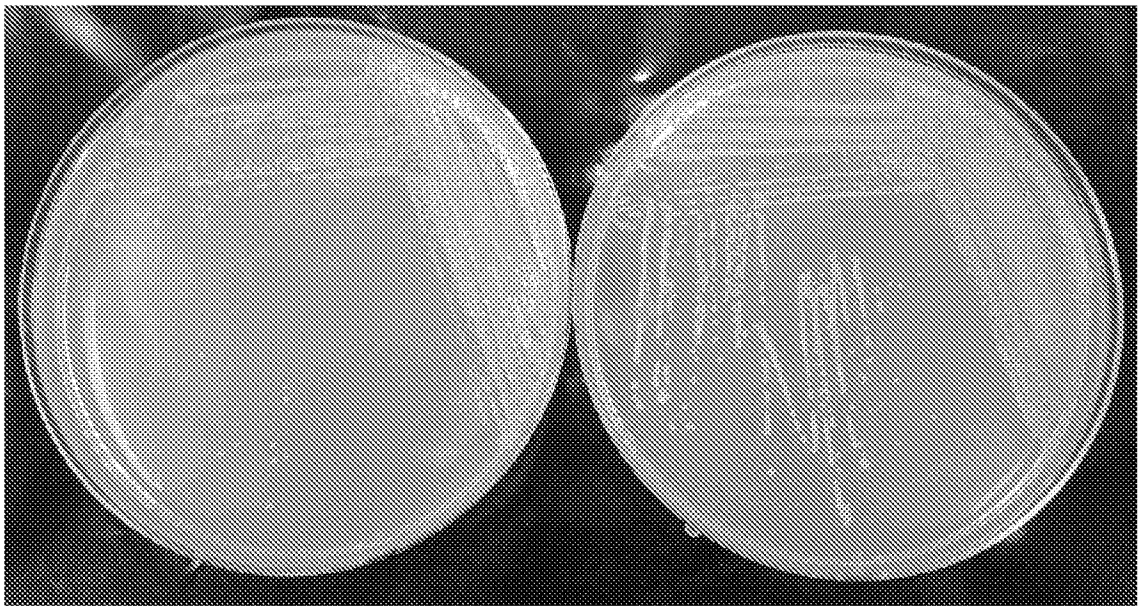


FIG. 8B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/23026

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 1/00, C12N 1/20, C12N 1/26 (2015.01)
CPC - C12N 1/20, C12N 15/70, C12N 1/26
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12N 1/00, C12N 1/20, C12N 1/26 (2015.01)
CPC: C12N 1/20, C12N 15/70, C12N 1/26

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/243, 435/252.33, 435/248

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWest, PatBase, Google Patents, Google Scholar: E.choli, Escherichia coli, bacteria, probiotic, culture, media, medium, inoculat*, buffer, liquid growth, MOPS (3-(N-morpholino)propanesulfonic acid), Tricine, Iron Sulfate, Ammonium Chloride, Potassium Sulfate, Calcium Chloride Monohydrate, Magnesium Chloride, Sodium Chloride, Ammonium Molybdate,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y -- A	TRAUTNER et al., PRE-INOCULATION OF URINARY CATHETERS WITH ESCHERICHIA COLI 83972 INHIBITS CATHETER COLONIZATION BY ENTEROCOCCUS FAECALIS, Journal of Urology, January 2002, Vol. 167, NO. 1, pages 375-379, Abstract, pg 2, para 3	1-15, 45 --- 16
Y -- A	US 2010/0310713 A1 (VIEBKE et al.) 09 December 2010 (09.12.2010); para [0011], [0023], [0066], [0071]	1-15, 45 --- 16
Y	US 2013/0195823 A1 (SCATIZZI et al.) 01 August 2013 (01.08.2013); para [0069]	9-10
Y	WO 2009/005704 A1 (KIZER) 08 January 2009 (08.01.2009); para [0070], [0074]	11-12
Y	US 5,536,645 A (JAY) 16 July 1996 (16.07.1996); abstract; col 2, ln 63-65	2, 3, 13, 15
A	US 2010/0279331 A1 (MORIYAMA et al.) 04 November 2010 (04.11.2010); para [0292], [0295], [0306]	16
A	US 2013/0302783 A1 (HAYASHIZAKI et al.) 14 November 2013 (14.11.2013); para [0047], [0069]	16
A	US 2013/0211310 A1 (BOMMARITO et al.) 15 August 2013 (15.08.2013); para [0115]	16
A	US 2013/0029415 A1 (NAOYA et al.) 31 January 2013 (31.01.2013); para [0123]	16
A	US 2012/0045432 A9 (YU et al.) 23 February 2012 (23.02.2012); para [0823]; [1107]	16
A	US 2006/0035287 A1 (KROLL et al.) 16 February 2006 (16.02.2006); para [0020]	16

☒ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
11 August 2015 (11.08.2015)

Date of mailing of the international search report
21 AUG 2015

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/23026

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2013/0323808 A1 (GOKARN et al.) 05 December 2013 (05.12.2013); para [0411]	16
A	US 2014/0051841 A1 (ALLEN et al.) 20 February 2014 (20.02.2014); para [0026], [0101]	16
A	US 2011/0287500 A1 (URANO et al.) 24 November 2011 (24.11.2011); para [0270]	16
A	US 2012/0115180 A1 (LAHTEENMAKI et al.) 10 May 2012 (10.05.2012); para [0042]	16
A	US 2007/0118916 A1 (PUZIO et al.) 24 May 2007 (24.05.2007); para [0003], [0006], [0506], [0563], [1095], [5965], [7824]	16
A	US 4,597,966 A (ZOLTON et al.) 01 July 1986 (01.07.1986) col 4, ln 62- col 5, ln 7	16
A	US 2013/0267680 A1 (OVAA et al.) 10 October 2013 (10.10.2013); [0129]	16
A	US 2013/0122541 A1 (LYNCH et al.) 16 May 2013 (16.05.2013); para [0640], Table 67	16
A	Proctor et al "Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections" NATURE REVIEWS MICROBIOLOGY VOLUME 4 pg 295-305, APRIL 2006, abstract, pg 295 col 1, para 1	1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/23026

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 19, 24, 25, 40-44, 46-67
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-16, 45, directed to a method of culturing probiotic bacteria.

Group II, claims 17, 18 and 20-23, 26-30, directed to a method of preparing a medical lubricant gel comprising probiotic small colony variant (SCV) bacterial cells, or a frozen or freeze-dried composition.

Group III, claims 31-39, directed to a method of forming a biofilm on a medical device or of administering SCV bacterial cells to a subject.

*****Continued in Supplemental Box*****

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-16, 45

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/23026

Continuation of Box No. III:

The inventions listed as Groups I-III do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special technical features
Group I has the special technical feature of a method of culturing probiotic bacteria, an E. coli SCV bacterium, in a defined liquid growth medium, that is not required by any other Groups.

Group II has the special technical feature of an aqueous fluid mixture comprising probiotic SCV bacterial cells, gelling agent and protective agent, that is not required by any other Groups.

Group III has the special technical feature of a method of forming a biofilm on a medical device or of administering SCV bacterial cells to a subject, that is not required by any other Groups.

Common technical features:
Groups I-III share the common technical feature of small colony variant (SCV) bacterium. Groups I and II further share a probiotic SCV liquid culture. Groups II and III further share the common features of a freeze-dried preparation comprising SCV bacterial cells, a pharmaceutically acceptable gelling agent, and a pharmaceutically acceptable first protective agent. However, this shared technical feature does not represent a contribution over prior art, because this shared technical feature is obviated by the article entitled "PRE-INOCULATION OF URINARY CATHETERS WITH ESCHERICHIA COLI 83972 INHIBITS CATHETER COLONIZATION BY ENTEROCOCCUS FAECALIS" by *Trautner et al. (hereinafter 'Trautner'), in view of US 2010/0310713 A1 to Viebke et al., (hereinafter* Viebke).

Trautner teaches an E. coli small colony variant (SCV) liquid culture (pg 2, para 3, E. coli 83972) bacteria were retrieved from the trypticase soy agar plates and cultured overnight in trypticase soy broth (Difco, Sparks, Maryland)).
Viebke teaches formulating a probiotic bacterium liquid culture (para [0011], [0071]), comprising a freeze-dried preparation comprising bacterial cells (para [0011]), a pharmaceutically acceptable gelling agent (para [0066] 'Examples of polysaccharides could be carrageenan, pectin, alginate'), and a pharmaceutically acceptable first protective agent (para [0011] 'the addition of gum Arabic to the probiotic composition provides protection for the probiotic culture'). It would have been obvious to one of ordinary skill in the art to have formulate the E. coli SCV liquid culture of Trautner according to the teachings of Viebke, and thus provide a pharmaceutic composition comprising E. coli SCV bacterium that is suitable to coat catheter and administer to a human subject.

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Groups I-III lack unity of invention under PCT Rule 13.

Note: continued from no. 4 above: Claims 19, 24, 25, 40-44, 46-67 are held unsearchable because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).