

Bacterial secretome for use in the treatment of skin lesions

5 FIELD OF THE INVENTION

The present invention relates to a novel use of a bacterial secretome in the field of the treatment of skin lesions and more particularly of wound healing. The invention also relates to cosmetic or dermatological compositions comprising such a bacterial secretome as active agent.

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PRIOR ART

Wound healing is a complex and dynamic biological process that involves the interaction of many local and systemic factors in normal tissue repair. Healing progresses in three interdependent phases: haemostasis and inflammation, proliferation and remodelling (General principles of wound healing. Witte MB, Barbul A. Surg Clin North Am. 1997 June; 77(3):509–28.). Proliferation involves three clearly observable processes: granulation, contraction and re-epithelialization.

During granulation, cells involved in the repair process are observed to proliferate and migrate toward the wound bed. For example, macrophages, fibroblasts and endothelial cells are found there. Macrophages steadily release chemotactic factors and growth factors. Fibroblasts construct the new cellular matrix necessary to cell growth at the base of the wound. This scaffolding supports cell migration. Lastly, endothelial cells trigger the formation of vascular buds which will establish new capillaries, thereby restoring perfusion and ensuring the supply of oxygen and of nutrients essential to the metabolic activity of cells in the wound.

Wound contraction is a mechanism of reducing wound size, and fibroblasts play a leading role in this contraction.

Re-epithelialization consists in the regeneration of an epidermis that covers a wound to form an effective barrier against the external environment, capable of becoming pigmented and of recovering its sensory and immune functions. It thus involves the cellular processes of keratinocyte migration and proliferation, but also the

differentiation of this neo-epithelium and the restoration of a basal membrane connecting the dermis and the epidermis. When the migration of basal cells towards the centre of the wound enables the two wound edges to meet, a wave of cell mitosis occurs to fill the spaces left by the migration and to provide cells for the epithelial tissue in
 5 three-dimensional regeneration.

The proliferation stages of keratinocyte cells, fibroblasts and endothelial cells can be regarded as one of the functional phenomena showing the healing activity of an active agent. Increased fibroblast proliferation would participate in the healing of a deep wound (reaching the dermis), whereas increased keratinocyte proliferation would
 10 participate in re-epithelialization.

Healing also poses cosmetic problems when healing defects, or poor healing, cause unsightly and mostly permanent visible marks such as thick scars or keloids.

All cosmetic and dermatological surgical procedures, as well as pathological and nonmedical traumatic lesions (scratches, cuts, scrapes, burns), leave scars. One of the
 15 principal skills of plastic surgeons is to control scar formation.

Pressure, for example, has been used for many years for burn scars and recently has been combined with silicone sheeting laid directly on the scar.

There remains a need to propose novel compositions for promoting the healing of damaged skin and for improving the cosmetic appearance of scars.
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For the first time, and surprisingly, the applicant has shown the beneficial properties in tissue regeneration and in healing of skin lesions of a bacterial secretome derived from a bacterial strain (or bacterium), LMB64, isolated from groundwater.

This bacterium was described by the Applicant in patent application
 25 WO2012/085182. More particularly, the bacterium was described as such for its anti-inflammatory activities. Even more particularly, extracts named S0, E0 and ES0 were described and exemplified, said extracts consisting of the culture supernatant separated from the biomass, the biomass of lysed cells, and the supernatant after incubation of the culture at basic pH for several hours, respectively. Only the pharmacological activities
 30 of extracts E0 and ES0 were tested, and it was shown that these extracts E0 and ES0 were able to induce cytokines and the maturation of Langerhans cells (for E0) as well as activation of TLR2/TLR4/TLR5, antagonists of PARs, and induction of antimicrobial

peptides (for ES0). These results suggested the use of such extracts in the treatment of inflammatory diseases such as pruritis, psoriasis, eczema or atopic dermatitis.

Against all expectations, the Applicant shows herein that a secretome consisting not of the same components as the above-mentioned extracts ES0 and E0, but mainly consisting of the biomolecules secreted and externalized into the culture supernatant by said bacterium LMB64, has surprising healing properties.

DETAILED DESCRIPTION

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According to a first embodiment (1), the present invention relates to a bacterial secretome of a non-pathogenic Gram-negative bacterium belonging to the class Betaproteobacteria, subfamily Neisseriaceae, containing a 16S rRNA comprising sequence SEQ ID No. 1, or any sequence having at least 80% identity with sequence SEQ ID No. 1, for use in the treatment of skin lesions,

said secretome being likely to be obtained, or obtained, by a process comprising the steps of:

- a) culturing said bacterium in a culture medium and under conditions suitable for its growth,
- b) liquid/solid separation and recovery of the liquid phase,
- c) obtaining said secretome comprising the biomolecules secreted by said bacterium in the liquid phase.

According to a second embodiment of the present invention, the bacterial secretome for use in the treatment of skin lesions according to the invention is characterized in that a basic buffer is added to the liquid phase obtained in step b) and that the buffered liquid phase obtained is optionally left in contact for 1 to 7 h, preferably at a temperature between 1 and 6°C.

Bacterium LMB64 has been characterized and defined as belonging to the class Betaproteobacteria, subfamily Neisseriaceae, and probably a novel genus not yet defined. Analysis of the gene sequence encoding 16S ribosomal RNA (rRNA) made it possible to place this bacterium close to the genera *Chromobacterium*, *Paludimonas*, *Lutelia* and *Glubenkiana*, with which it shares 95% sequence similarity.

This non-pathogenic bacterium is Gram-negative and was isolated from groundwater.

More particularly, bacterium LMB64 is rod-shaped with a length of roughly 2.3 μm (± 0.3) and a width of roughly 1.0 μm (± 0.1). A characteristic feature of this bacterium is the presence of a polar flagellum.

The gene encoding 16S rRNA has been almost completely sequenced (1487 bp, corresponding to sequence SEQ ID No. 1). Bacterium LMB64 has a circular plasmid of 10948 bp. This plasmid was completely sequenced, and the sequence is represented in sequence SEQ ID No. 2.

According to another embodiment, a bacterium from which the secretome of the invention is derived contains at least one plasmid comprising sequence SEQ ID No. 2, or any sequence having at least 80% identity with sequence SEQ ID No. 2, advantageously at least 85%, at least 90%, at least 95%, even at least 97% and more preferentially still at least 98% identity with sequence SEQ ID No. 2.

In addition, a bacterium from which the secretome is derived is a non-pathogenic Gram-negative bacterium belonging to the class Betaproteobacteria, subfamily Neisseriaceae, said bacterium containing a 16S rRNA comprising sequence SEQ ID No. 1, or any sequence having at least 80%, even at least 90%, at least 95%, at least 97% identity with sequence SEQ ID No. 1 and said bacterium containing at least one plasmid comprising sequence SEQ ID No. 2, or any sequence having at least 80% identity with sequence SEQ ID No. 2, advantageously at least 85%, at least 90%, at least 95%, even at least 97% and more preferentially still at least 98% identity with sequence SEQ ID No. 2.

By way of example, such a bacterium is represented by strain LMB64, which was deposited in the name of the Applicant in the *Collection Nationale de Cultures de Microorganismes* (CNCM), Institut Pasteur, Paris, on 8 April 2010 under number I-4290.

Having this genotypic information, combined with the characteristics concerning growth in sulphur-free medium and the non-filamentous nature of this bacterium, a person skilled in the art would have no difficulty in finding/identifying another bacterium allowing a secretome of the invention to be obtained. Such an identification of another bacterium, of course slightly genotypically different but possessing all the

phenotypic criteria of the invention in terms of the secretome, can be made after a selection study, which is in no case insurmountable, and this based on the information contained in the present application and that contained in application WO2012/085182, combined with the general knowledge of the person skilled in the art.

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In the context of the present invention, “percentage identity” between two nucleic acid sequences refers to a percentage of identical nucleotides between two sequences to be compared, obtained after the best alignment (optimal alignment), this percentage being purely statistical and the differences between the two sequences being distributed randomly and over their entire length. Comparisons of sequences between two nucleic acid sequences are usually made by comparing these sequences after having aligned them in an optimal manner, wherein said comparison may be made per segment or per “comparison window”. The optimal alignment of the sequences for the comparison can be performed, in addition to manually, by means of the local homology algorithm of Smith and Waterman (1981) [Ad. App. Math. 2:482], by means of the local homology algorithm of Needleman and Wunsch (1970) [J. Mol. Biol. 48:443], by means of the similarity search method of Pearson and Lipman (1988) [Proc. Natl. Acad. Sci. USA 85:2444], or by means of computer software using these algorithms (GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI, or the BLAST N or BLAST P comparison software).

The percentage identity between two nucleic acid sequences is determined by comparing these two aligned sequences in an optimal manner, wherein the nucleic acid sequence to be compared may include additions or deletions in relation to the reference sequence for an optimal alignment between these two sequences. Percentage identity is calculated by determining the number of positions for which the nucleotide is identical between the two sequences, dividing this number of identical positions by the total number of positions in the comparison window, and multiplying the result obtained by 100 to obtain the percentage identity between these two sequences.

For example, the BLAST program “BLAST 2 sequences” (Tatusova et al., “Blast 2 sequences – a new tool for comparing protein and nucleotide sequences,” FEMS Microbiol Lett. 174:247–250), available at

http://www.ncbi.nlm.nih.gov/gorf/b12.html, may be used with the default parameters (in particular for the parameters “open gap penalty”: 5, and “extension gap penalty”: 2; with the selected matrix being for example the “BLOSUM 62” matrix proposed by the program). The percentage identity between the two sequences to be compared is calculated directly by the program. It is also possible to use other programs such as the “ALIGN” or “Megalign” software (DNASTAR).

A bacterium of the invention includes bacterium LMB64 which contains at least one plasmid comprising sequence SEQ ID No. 2, or any sequence having at least 80%, preferably 85%, 90%, 95% and 98% identity with said sequence SEQ ID No. 2. Other features of said bacterium LMB64 will be detailed below in the examples.

Moreover, bacterium LMB64 was deposited in the name of the Applicant in the *Collection Nationale de Cultures de Microorganismes* (CNCM), Institut Pasteur, Paris, on 8 April 2010 under number I-4290.

According to another embodiment, the present invention also relates to a bacterial secretome for use in the treatment of skin lesions according to the second embodiment, characterized in that the basic buffer is selected from Tris buffer, arginine buffer and mixtures thereof.

In another embodiment, the invention relates to a bacterial secretome for use in the treatment of skin lesions according to one of the preceding embodiments, characterized in that step b) of liquid/solid separation is followed by a step b') of clarification of the liquid phase or of the buffered liquid phase by filtration before step c).

In another embodiment, the invention relates to a bacterial secretome for use in the treatment of skin lesions according to the preceding embodiment, characterized in that filtration is carried out with a 0.2 μm cut-off.

According to another embodiment, the invention also relates to a bacterial secretome for use in the treatment of skin lesions according to one of the preceding

embodiments, characterized in that said biomolecules consist of the peptides, proteins and secondary metabolites secreted by said bacterium.

5 It is also another embodiment of the present invention to provide a bacterial secretome for use in the treatment of skin lesions according to one of the preceding embodiments, characterized in that the bacterium contains at least one plasmid comprising sequence SEQ ID No. 2, or any sequence having at least 80% identity with sequence SEQ ID No. 2.

10 According to another embodiment, the invention also relates to a bacterial secretome for use in the treatment of skin lesions according to one of the preceding embodiments, characterized in that the bacterium is the bacterium deposited with the CNCM on 8 April 2010 under number I-4920.

15 Generally, the term “secretome” is used to describe all the biomolecules secreted and excreted into the culture medium and solubilized in this medium, by a cell, a tissue or an organism. In the present case, secretome must be understood to refer to all the biomolecules secreted and externalized by bacterium LMB64, and this with no modification, deterioration or lysis of the bacterial cell. More particularly, these
20 biomolecules consist mainly of the proteins, peptides and secondary metabolites secreted and externalized by the bacterium into the culture medium.

Bacteria multiply by binary fission, i.e., each bacterium grows and then divides into two daughter cells separated by a division septum formed by the cell wall. During division, DNA and other components are duplicated. Various enzymatic systems of
25 synthesis and degradation take part in cell division.

Bacterial growth is the ordered increase of all the components of the bacterium. It results in an increase in the number of bacteria. During growth, there is, on the one hand, a depletion of nutrients from the culture medium and, on the other hand, an enrichment in biomolecules secreted and excreted into the culture medium by the
30 bacterium and solubilized in this medium, as well as metabolic by-products.

The expression “culture medium” or “medium” must be understood to refer to any medium comprising at least the nutrients necessary for bacterial growth and

multiplication. The bacteria may be grown in liquid, solid or semi-liquid medium. Preferably, the culture medium is a liquid medium allowing recovery of the culture supernatant containing the secretome.

5 A suitable culture medium contains nutrients which promote bacterial growth and multiplication. Generally, a suitable culture medium will comprise water, a carbon source, a nitrogen source, and salts. By way of illustration, a particular exemplary culture medium is described below in the examples.

10 The expression “secondary metabolites” must be understood to refer to the small molecules that a bacterium of the invention, in particular bacterium LMB64, produces, secretes and excretes into the culture medium. By way of illustration, such secondary metabolites may be soluble molecules such as bacteriocins, non-ribosomal peptides, siderophores, lipopeptides or polyketides, or molecules such as terpenes, pyrazines, indoles, or sulphide derivatives. The proteins present in the secretome have sizes between 10 kDa and 100 kDa, preferentially between 18 kDa and 98 kDa, and
15 preferably a majority protein whose size is roughly 38 kDa.

In practice, the secretome of a bacterium of the invention, for example and in particular bacterium LMB64, can be obtained from a culture in a culture medium allowing the growth, development and multiplication of the bacterium (as for example
20 that described below) by simply recovering the culture supernatant containing the secreted biomolecules making up the secretome of the invention. In concrete terms, owing to the fact that these biomolecules are secreted and externalized by the bacterium and solubilized in the medium, it is not necessary to perform a step of treating said bacteria (mechanical or chemical, inducing total or partial cell lysis or release of
25 proteins or components of the wall, the membrane or the periplasmic space). The components of the culture supernatant (hereafter the secretome) can be recovered from the insoluble solid cellular components by any liquid/solid separation means. More particularly, it is therefore possible, by the techniques known to persons skilled in the art, to isolate the liquid fraction containing the solubilized biomolecules from the solid
30 fraction containing mostly whole cells and cell debris comprising surface proteins and/or proteins located in the periplasmic space of the bacterium.

By way of illustration, liquid/solid separation can be performed by a technique selected from: centrifugation, sedimentation, filtration, ultrafiltration, decantation, draining, for example.

5 Preferably, liquid/solid separation is performed by centrifugation of the bacterial culture in order to separate the solid phase, i.e., the pellet, containing cells and cell debris; and the liquid phase, i.e., the supernatant, comprising the soluble molecules, i.e., the secretome.

10 The supernatant, i.e., the liquid phase, can be used as such, or after simple filtration in order to remove the cell debris possibly present and to sterilize it. It may also be envisaged to concentrate it, for example by diafiltration. The proteins making up the secretome can also be recovered by precipitation with ammonium sulphate.

15 As indicated above, the process for preparing a bacterial secretome of the invention is characterized in that a basic buffer is added to the liquid phase obtained in step b) and that the buffered liquid phase obtained is optionally left in contact with such a buffer for 1 to 7 h, in particular at low temperature, preferably at a temperature between 1 and 6°C.

20 The addition of a basic buffer to the supernatant improves the solubility and/or stability of proteins and peptides in solution.

 Preferably, this basic buffer is a Tris buffer or an arginine buffer or a Tris–arginine mixture. Preferably it is a Tris–arginine buffer.

25 The amount of basic buffer, preferentially Tris–arginine, added to the liquid phase is such that the buffered liquid phase obtained has an arginine concentration between 100 mM and 500 mM and/or a Tris concentration between 10 mM and 90 mM.

 The pH of the buffered liquid phase may be between 8 and 12, and preferably between 9 and 11.

30 The use of a basic buffer (Tris, arginine or Tris–arginine) allows stabilization of the proteins in solution and avoids their aggregation over a long storage period.

A preferred method for culturing the bacterium of the invention, in particular strain LMB64, can comprise three steps.

- A first inoculum can be prepared in an Erlenmeyer flask in a suitable medium containing mineral, protein and carbohydrate substances appropriate to this pre-culture.
- A second pre-fermentation culture in batch mode in a second medium similar or identical to the first medium (or any equivalent medium) is then prepared.
- Lastly, fermentation in fed-batch mode in a medium identical or similar to the second medium in order to obtain a high cell density.

A centrifugation step then allows removal of the cells and cell debris and recovery of the culture supernatant.

This buffered supernatant, or buffered liquid phase, can then be clarified by filtration.

As indicated above, step b) of liquid/solid separation is followed by a step b') of clarification of the liquid phase or of the buffered liquid phase, for example by filtration, before step c).

Filtration may be performed by any suitable means allowing clarification of the liquid phase or, if applicable, of the buffered liquid phase. Such clarification by filtration removes the suspended particles not removed during the liquid/solid separation step, and is directed at producing a clear secretome.

Filtration can be performed by any filtration, ultrafiltration or diafiltration means.

Advantageously, filtration is performed by filtration through a filter or a cartridge filter having a 0.4 μm cut-off, preferentially 0.2 μm . In this case, the bacterial secretome is characterized in that the secreted peptides, proteins and secondary metabolites have a size of 0.2 μm or less.

Preferably, non-electrostatically-charged filters or prefilters can be used in order to avoid any absorption of the biomolecules responsible for all or part of the activity.

5 The exact nature of the media and of the various steps will be described in greater detail in the examples. It must be understood that any modification of the process, of the media, or of the sequences of steps which would be obvious to a person skilled in the art with regard to the present description must be regarded as within the scope of the present patent application.

10 As mentioned above, the invention also concerns, in another embodiment, a bacterial secretome for use in the treatment of skin lesions according to one of the preceding embodiments, characterized in that the skin lesions are selected from the group consisting of abrasions, burns, sunburns, scratches, cuts, scrapes, stitches.

15 The invention also relates to a bacterial secretome for use in the treatment of skin lesions according to one of the preceding embodiments for use for the mitigation of healing defects.

20 The invention also concerns, in another embodiment, a bacterial secretome for use in the treatment of skin lesions according to one of the preceding embodiments, characterized in that the skin lesions follow trauma, surgery, a dermatological procedure, or a cosmetic medicine procedure.

25 Advantageously, the invention also relates to a bacterial secretome for use in the treatment of skin lesions according to one of the preceding embodiments, characterized in that it is combined with a copper salt or a zinc salt.

30 In another embodiment, the invention also relates to a bacterial secretome for use in the treatment of skin lesions according to one of the preceding embodiments, characterized in that the treatment of skin lesions consists in improving healing and skin repair.

The invention further concerns, in another embodiment, a method of cosmetic treatment for mitigating skin-healing defects comprising the application of an effective amount of a composition containing an effective amount of a bacterial secretome as defined in one of the preceding embodiments in combination with a cosmetically acceptable excipient and formulated in a form suitable for topical application.

Advantageously, the invention also relates to a bacterial secretome for use in the treatment of healing defects according to one of the preceding embodiments, characterized in that it is combined with a copper salt and/or a zinc salt.

Therefore, the invention further concerns, in another embodiment, the use of a bacterial secretome as defined in one of the preceding embodiments for the mitigating cosmetic treatment of skin-healing defects. According to a particular embodiment, the secretome of the invention is used in combination with a copper salt and/or a zinc salt.

In another embodiment, the invention relates to a dermatological composition containing a bacterial secretome as defined in one of the previous embodiments and a dermatological excipient suitable for topical application. In an advantageous embodiment, the dermatological composition further includes a zinc salt and/or a copper salt.

In another embodiment, the invention further relates to a dermatological composition according to one of the preceding embodiments for use in the treatment of skin lesions. In an advantageous embodiment, the dermatological composition further includes a zinc salt and/or a copper salt.

In another embodiment, the invention relates to a cosmetic composition containing a bacterial secretome as defined in one of the preceding embodiments and a cosmetic excipient suitable for topical application. The cosmetic composition can further include a zinc salt and/or a copper salt.

Lastly, in another embodiment, the present invention relates to the use of a cosmetic composition according to a preceding embodiment for the mitigating cosmetic treatment of skin-healing defects.

According to an embodiment, the invention relates to a bacterial secretome as defined in one of the preceding embodiments, for topical use for promoting fibroblast proliferation.

5 According to an embodiment, the invention relates to a bacterial secretome as defined in one of the preceding embodiments, for topical use for promoting keratinocyte proliferation and/or migration.

 According to an embodiment, the invention relates to a bacterial secretome as defined in one of the preceding embodiments, for topical use for treating skin lesions

10 According to an embodiment, the invention relates to a bacterial secretome as defined in one of the preceding embodiments, for topical use for improving skin healing and recovery.

 The invention also relates to a composition for accelerating skin repair in order to restore skin integrity and quality, comprising as active principle the secretome defined in one of the preceding embodiments. More advantageously, the composition

15 can further include a copper salt and/or a zinc salt.

 Preferably, the concentration of the secretome in a composition of the invention is between 0.05% and 10%. More preferably, it is between 0.1% and 5%.

20 The composition of the invention further includes one or more conventional dermatologically and/or cosmetically compatible excipients known to persons skilled in the art in order to obtain a composition for topical application, such as emulsifiers, thickeners, gelling agents, water binders, spreading agents, stabilizers, dyes, fragrances and preservatives.

25 In the present invention, the expression “dermatologically or cosmetically acceptable” is intended to mean that which is useful in the preparation of a dermatological or cosmetic composition, which is generally safe, nontoxic and neither biologically nor otherwise undesirable and which is acceptable for dermatological or cosmetic use, and in particular dermatological or dermo–cosmetic use, in particular by

30 topical application.

The compositions according to the invention are advantageously for topical application, in particular to the skin.

5 The composition of the invention can be prepared in the form of an emulsion, a dispersion, or an aqueous or oily lotion.

In another particular embodiment, the dermatological or cosmetic composition of the invention includes at least one other active principle, more particularly at least one moisturizing active principle.

10 This other active principle may thus particularly be selected from the group consisting of:

- Well-known healing or restorative agents: panthenol, madecassoside, allantoin, aloe vera, honey;
- Anti-infective agents: copper-zinc-type trace elements, honey
- 15 • Moisturizing and nourishing agents: glycerine, shea butter, natural waxes, hyaluronic acid, fatty acids
- Soothing agents: allantoin, bisabolol, panthenol, enoxolone
- UV filters.

Preferably, the second active principle will be a copper-zinc-type trace element.
20 In this preferred embodiment, the composition of the invention will thus include the secretome and the copper-zinc-type trace element.

As mentioned, a dermatological or cosmetic composition of the invention comprising the secretome may include a copper salt or a zinc salt. Preferably, the copper salt will be copper sulphate. Advantageously, the zinc salt will be selected from zinc
25 oxide and zinc sulphate.

The amount of copper sulphate in a composition of the invention may vary between 0.05 and 0.5 wt% of the composition, preferentially between 0.1 and 0.25 wt% of the composition, more preferentially roughly 0.2 wt% of the composition.

30 The amount of zinc sulphate in a composition of the invention may vary between 0.05 and 0.5 wt% of the composition, preferentially between 0.1 and 0.4 wt% of the composition, more particularly between 0.1 and 0.2 wt% of the composition.

The amount of zinc oxide in a composition of the invention may vary between 0.5 and 10 wt% of the composition, preferentially between 1 and 5 wt% of the composition, more particularly roughly 2 to 4 wt% of the composition.

5 An embodiment of the invention envisages a composition according to one of the preceding embodiments characterized in that it further includes at least one other active principle selected from healing agents, anti-infectives, moisturizing agents, soothing agents and mixtures thereof. According to another particular embodiment, at least one other active principle will be selected from moisturizing agents, healing
10 agents, soothing agents and mixtures thereof, preferably moisturizing agents. Such an active principle will be used preferably in dermatological compositions.

The present invention also relates to a method for treating and healing skin lesions comprising the administration, preferably topical, of an effective amount of a composition containing as active principle a secretome of the invention.

15 The compositions according to the invention are in particular for the care of skin having sustained lesions.

Skin lesions can appear following trauma or medical or surgical procedures, whether invasive or non-invasive, curative or for cosmetic purposes.

Invasive medical or surgical procedures include: surgical procedures (excision, shaving)
20 with or without stitches, cryotherapy, laser ablation, moderate or strong chemical peels, stitches, mesotherapy or curettage, for example.

Post-traumatic skin lesions, for example following mild external damage to the skin, include cuts, scratches, scrapes and superficial burns or sunburn, for example.

25 Superficial non-invasive medical procedures requiring a healing product that accelerates skin recovery and is suitable for long-term use (until the skin is completely repaired) include mild chemical peels, laser hair removal, and superficial vascular (rosacea) treatments, for example.

30 The treatment of skin and mucous membrane lesions of the invention may particularly include the treatment of cuts, stitches, abrasions, scrapes, scratches, scars following surgery or a cosmetic dermatology procedure, superficial burns, sunburn.

The present invention further relates to the use of a cosmetic composition of the invention for improving healing and skin repair and in particular for mitigating healing defects.

5 The invention will be better understood by reading the examples below which illustrate the invention without limiting its scope.

The examples refer to the following figures

FIGURE 1: SDS–PAGE gel of secretome proteins.

10 Lane 1 (M, molecular weight markers, Sea Blue).

Lanes 2 to 5: (Sec, secretome before addition of arginine buffer) – 10 µl, 15 µl, 20 µl and 30 µl loads, respectively.

Lanes 6 to 9: (Sec + Arg, secretome after addition of arginine buffer) – 10 µl, 15 µl, 20 µl and 30 µl loads, respectively.

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FIGURE 2: % stimulation of proliferation (mean $\pm$ SEM) of normal human fibroblasts treated with the various products compared with control cells (n=3 donor pools).

20 **FIGURE 3:** % stimulation (mean $\pm$ SEM) of migration of the HaCaT keratinocyte cell line treated with the various compounds compared with control cells (n=2).

FIGURE 4: Wound re–epithelialization (mean $\pm$ SEM) after application for 48 h on explants of pig ears of the various products compared with a reference restorative cream (n=10 independent donors).

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Example 1: Culture of bacterium LMB64

By way of non–limiting example, preferred culture media contain ammonium chloride, magnesium sulphate and yeast extract. It should also be noted that, as arises from application WO2012/085182, among others, other similar media may be used and must thus be regarded as forming an integral part of the present description. Any adaptation by persons skilled in the art must also be regarded as part of the invention.

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An exemplary culture process is described below. It should be recalled here that this example is for illustrative purposes only and must in no way be regarded as limiting.

5 Strain LMB64 is grown in three steps, namely a first inoculum, a pre-culture (or pre-fermentation) in batch mode and finally a culture but in fed-batch mode (addition of glucose).

Inoculum: A tube of WCB LMB64 is used to inoculate an Erlenmeyer flask containing 1000 mL of sterile medium. The Erlenmeyer flask is then placed in the incubator shaker and shaken. When the cell density of the broth is sufficient, the culture
10 is stopped. The cells are then cooled until transfer to the pre-fermenter.

Pre-culture: The pre-fermenter is then filled with roughly 16 L of medium and then fully sterilized.

Two satellite flasks are connected to the pre-fermenter after sterilization of the tank and then of the addition blocks:

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- a flask containing a sterile 50% glucose solution. This solution (Glucose batch pre-culture) is immediately transferred to the culture medium to attain the initial glucose concentration of 20 g/L.

- The Erlenmeyer flask containing the inoculum described above in the

Inoculum step is inoculated in the pre-fermenter.

20 The pre-culture is launched and then regulated automatically. By way of example, the following parameters may be mentioned: Temperature, Shaking speed, Pressure, Air flow rate, or PO₂.

25 Cell growth is monitored by measurement of the optical density at 620 nm. The pre-culture is stopped by cooling when it reaches a sufficient density.

Culture: The fermenter is then filled with 127 L of medium adjusted to pH 7.0 and then fully sterilized. Three satellite flasks are used:

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- a flask containing a sterile glucose solution. This solution is immediately transferred to the culture medium to attain the initial glucose concentration of 20 g/L.

- a flask of antifoam. This antifoam will be added automatically during the culture to control the level of foam in the tank.
- a flask of fed-batch glucose. This solution will be added during the culture to promote cell growth.

5 The culture is launched and then regulated automatically. By way of example, the following parameters may also be mentioned: Temperature, pH, Shaking, Pressure, air flow rate, PO₂.

10 After exhaustion of the glucose initially present in the medium (rise in PO₂), addition of the fed-batch glucose solution is triggered, thus enabling high-density cell growth. Fermentation is stopped after total consumption of the glucose. At this stage, the fermentation must be automatically cooled. Throughout the culture, cell growth is monitored by measurement of the optical density at 620 nm. The amount of dry biomass (g/L) obtained at the end of the culture is determined using a weight method.

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Example 2: Extraction of the secretome

The example below is given by way of illustration of a preferred embodiment, but must not be regarded as limiting.

20 The secretome is obtained, generally, after centrifugation of the result of the culture step in order to remove cells, surface proteins and proteins located in the periplasmic space of the bacterium. This centrifugation step is followed by an addition of a Tris-arginine basic buffer to the supernatant. A last step of filtration of the supernatant is also possible. Any adaptation by persons skilled in the art must also be regarded as part of the invention.

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30 **Centrifugation:** The transfer line from the fermenter to the centrifuge is sterilized. The fermentation must be then separated by continuous centrifugation on a centrifuge. Centrifugation is performed at 150 L/h (± 30 L/h) with a bowl rotation speed of 10900 ± 1000 rpm. The supernatant is collected in a container fitted with a disposable pouch. The weight of the supernatant is measured on the balance platform.

Addition of arginine: The Tris arginine extraction buffer is sterilized and then transferred to the disposable pouch containing the culture supernatant. The contact time necessary is between 1 and 7 hours. The target concentration of Tris and arginine after addition to the disposable pouch is roughly 0.3 M L-arginine and 20 mM Tris.

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The total volume after addition of the extraction buffer is roughly 240 L.

Filtration: Two filtration steps are performed on-line, in order to clarify the supernatant and to produce a clear, germ-free secretome. Filtration is controlled by the filtration/distribution system. The disposable depth-filtration cartridge is placed in its filter housing. All of the filtered product is recovered sterily in a container fitted with a disposable pouch. The pouch of filtered product is weighed on the balance platform and then stored at +5°C until distribution.

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Gel electrophoresis of secretome proteins: see Figure 1

Bis-Tris SDS-PAGE gel (4 – 12%), under denaturing and reducing conditions, detection by Coomassie Blue of the loads of various volumes of culture supernatants (10, 15, 20, 30 µl, respectively) before and after addition of arginine buffer. In both cases, various protein bands of between 14 and 100 kDa in size are observed, including a major band of roughly 38 kDa.

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Example 3: Effect of the secretome on proliferation of normal human fibroblasts

The technique used is that of incorporation of a nucleotide, 5-bromo-2'-deoxyuridine (BrdU), a thymidine analogue, in the DNA of S-phase cells, at 37°C. This technique allows quantification of the cells whose progression in the cell cycle is characteristic of a proliferative cell (S or DNA synthesis phase).

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Fibroblasts are seeded in a 96-well plate and then incubated for 72 h in the presence of the compounds to be tested, EGF as positive control and 0.4, 0.6 and 1% secretome. BrdU incorporation is performed during the last 24 h and is quantified using the BrdU ELISA kit (item no. 11647229001, Roche Diagnostics).

30

The % of the control is calculated according to the formula:

(Value / mean of the control) x 100

Stimulation in % is calculated according to the formula:

Mean Stimulation in % = (Mean (Value / mean of the control) x 100) – 100

SEM = standard dev / $\sqrt{n}$

- 5 Statistical analyses are performed using Student's *t*-test. The studies were carried out on 3 donor pools.

The positive control, EGF, stimulated fibroblast proliferation strongly and significantly and in a reproducible manner on the 3 donor pools (95% stimulation). This validates the experiments. The results show that the secretome tested at 0.4%, 0.6% and 10 1% significantly stimulated proliferation of normal human fibroblasts. Table 1 below summarizes the results of BrdU incorporation (mean $\pm$ SEM) by normal human fibroblasts (by ELISA) after 72 h of incubation in the presence of the tested compounds (n=3 donor pools). The results are shown in Figure 2.

15 **Table 1**

Compound	control	EGF 10 ng/ml	Secretome 0.4%	Secretome 0.6%	Secretome 1%
Mean % control	100	195	138	134	127
SEM	2	10	6	4	3
Mean % stimulation	0	95	38	34	27
SEM	2	10	6	4	3
Student's <i>t</i> -test	–	***	***	***	***

20 **Example 4: Effect of the secretome on migration of the HaCaT keratinocyte cell line**

The studies were performed using the Oris Cell Migration Assay (Platypus Technologies) according to the standard protocol recommended by the manufacturer. Chiefly, HaCaT (human keratinocyte cell line) cells are seeded in a 96-well plate. The Oris™ stoppers are removed and the compounds to be evaluated are added to the 25 culture medium. Incubation proceeds for 24 h to allow cell migration. A cell-permeable fluorescent tracer, calcein, is then added to allow visualization of the cells in the zone delimited by the stoppers.

Cell migration is evaluated by photographing the wells and measuring the migration surface area in mm².

The % of the control is calculated according to the formula:

$(\text{Value} / \text{mean of the control}) \times 100$

Stimulation in % is calculated according to the formula:

$\text{Mean Stimulation in \%} = (\text{Mean} (\text{Value} / \text{mean of the control}) \times 100) - 100$

5

SEM = standard dev / $\sqrt{n}$

Statistical analyses are performed using Student's *t*-test.

The studies are performed on 6 technical replicas and in n=2 independent experiments.

10 The positive controls, TGF- β and foetal calf serum (FCS), stimulated cell migration strongly and significantly and in a reproducible manner (51% and 176% stimulation, respectively). This validates the experiments. The results show that the secretome tested at 0.4%, 0.6% and 1% significantly stimulated HaCaT keratinocyte migration. Table 2 below summarizes the results of HaCaT cell migration after 24 h of
15 incubation with the various compounds (n=2). The results are shown in Figure 3.

Table 2

compound	control	FCS 10%	TGF- β 10 ng/ml	Secretome 0.4%	Secretome 0.6%	Secretome 1%
Mean % control	100	276	151	267	266	272
SEM	5	9	7	8	10	9
Mean % stimulation	0	176	51	167	166	172
SEM	5	9	7	8	10	9
Student's <i>t</i> -test	–	***	***	***	***	***

20

Example 5: Effect of the secretome on wound closing on an *ex vivo* model of healing

25 Skin explants of pig ears are maintained in survival conditions. Six-millimetre biopsies are performed at the centre of which a circular, 3 mm wound is made. The wound damages the entire epidermis and the upper dermis. The products to be evaluated are applied topically. Forty-eight hours later, the biopsies are frozen and haematoxylin–eosin staining is performed on sections. The kinetics of re-epithelialization is evaluated according to the following process:

30 1) Attribution of a score defined from 0 to 3 according to the protocol developed by J. Brandner (Pollok et al., 2010, J Cell Mol Med).

Precise measurement in μm of the extent of the re-epithelialized zone from the wound edge. Student's t -test is applied for significance.

2) The morphological appearance at the wound edge is also evaluated in order to assess tissue preservation quality.

5

Preparations of the products to be applied: the secretome was added at 0.4, 0.6 and 1 wt% to the same base formulation as that of a reference restorative cream (Cicalfate® cream) tested in parallel. Re-epithelialization is evaluated in comparison with this reference cream.

10

The results show a dose effect with the secretome on the progression of wound re-epithelialization. The formulation containing 1% secretome significantly accelerates the progression of wound closing compared with the reference restorative cream ($p=0.043$). Furthermore, morphological analysis of the tissues shows an excellent tolerance of the formulations containing the secretome. The results are shown in

15 Figure 4.

* * *

Exemplary composition: oil-in-water emulsion

20

Secretome according to Example 2: 0.1–5%

Glycerine: 1–30%

Hexylene glycol: 0.1 – 7%

Cetearyl glucoside: 0.1 – 1%

25

Cetearyl alcohol: 0.9 – 4%

Stearic acid: 0.5–4%

Glyceryl stearate: 0.1–4%

Vegetable oil: 0.1–10%

Water qs 100%

CLAIMS

1. A bacterial secretome of a non-pathogenic Gram-negative bacterium belonging to the class Betaproteobacteria, subfamily Neisseriaceae, containing a 16S rRNA comprising sequence SEQ ID No. 1, or any sequence having at least 80% identity with sequence SEQ ID No. 1, for use in the treatment of skin lesions, said secretome being obtained by a process comprising the steps of:
 - a) culturing said bacterium in a culture medium and under conditions suitable for its growth,
 - b) liquid/solid separation and recovery of the liquid phase,
 - c) obtaining said secretome comprising the biomolecules secreted by said bacterium in the liquid phase.
2. The bacterial secretome for use in the treatment of skin lesions according to claim 1, characterized in that a basic buffer is added to the liquid phase obtained in step b) and that the buffered liquid phase obtained is optionally left in contact for 1 to 7 h, preferably at a temperature between 1 and 6°C.
3. The bacterial secretome for use in the treatment of skin lesions according to claim 2, characterized in that the basic buffer is selected from Tris buffer, arginine buffer and mixtures thereof.
4. The bacterial secretome for use in the treatment of skin lesions according to one of claims 1 to 3, characterized in that step b) of liquid/solid separation is followed by a step b') of clarification of the liquid phase or of the buffered liquid phase by filtration before step c).
5. The bacterial secretome for use in the treatment of skin lesions according to claim 4, characterized in that filtration is performed with a 0.2 µm cut-off.

6. The bacterial secretome for use in the treatment of skin lesions according to one of claims 1 to 5, characterized in that said biomolecules consist of the peptides, proteins and secondary metabolites secreted by said bacterium.
- 5 7. The bacterial secretome for use in the treatment of skin lesions according to one of claims 1 to 6, characterized in that the bacterium contains at least one plasmid comprising sequence SEQ ID No. 2, or any sequence having at least 80% identity with sequence SEQ ID No. 2.
- 10 8. The bacterial secretome for use in the treatment of skin lesions according to one of claims 1 to 7, characterized in that the bacterium is the bacterium deposited with the CNCM on 8 April 2010 under number I-4920.
- 15 9. The bacterial secretome for use in the treatment of skin lesions according to one of claims 1 to 8, characterized in that the skin lesions are selected from the group consisting of abrasions, burns, sunburns, scratches, cuts, scrapes, stitches.
- 20 10. The bacterial secretome for use in the treatment of skin lesions according to one of claims 1 to 9, characterized in that the skin lesions follow trauma, surgery, a dermatological procedure, or a cosmetic medicine procedure.
- 25 11. The bacterial secretome for use in the treatment of skin lesions according to one of claims 1 to 10, characterized in that the treatment of skin lesions consists in improving healing and skin repair.
12. The bacterial secretome for use in the treatment of skin lesions according to one of claims 1 to 11, characterized in that it is combined with a copper salt and/or a zinc salt.
- 30 13. A dermatological composition containing a bacterial secretome as defined in one of claims 1 to 8 and a dermatological excipient suitable for topical application, for use in the treatment of skin lesions.

14. A dermatological composition containing a bacterial secretome as defined in one of claims 1 to 8, a copper salt and/or a zinc salt and a suitable dermatological excipient.
- 5 15. The dermatological composition according to claim 14, for use in the treatment of skin lesions.