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(54) Title: METHOD OF CULTURING SEGMENTED FILAMENTOUS BACTERIA IN VITRO

(57) Abstract: The present invention relates to an in vitro method of culturing a segmented filamentous bacterium strain, comprising co-culturing said segmented filamentous bacterium strain with a eukaryotic host cell, wherein the culture is performed at an O₂ level inferior to 5% in a rich tissue culture liquid medium containing bacterial medium components including iron. The present invention also relates to methods for genetically modifying a segmented filamentous bacterium strain comprising a step a culturing the strain in vitro.



METHOD OF CULTURING SEGMENTED FILAMENTOUS BACTERIA *IN VITRO*

The present invention relates to an *in vitro* method for growing segmented filamentous bacteria (SFB). The present invention also relates to methods for genetically modifying a SFB strain.

Segmented filamentous bacteria (SFB) or *Candidatus arthromitus* or *Candidatus savagella* or *Arthromitus immunis* or *Arthromitus muris* are gram-positive anaerobic, clostridia-related, spore-forming commensals found in the gut (distal ileum) of many vertebrate species, including mouse, rat, rabbit, birds (*e.g.*, chicken), fish, amphibian and probably humans (Klaasen *et al.*, 1992; Yin *et al.*, 2012). The size of the filament is comprised between 0.5 and 1000 μm in length. SFB produce intracellular offsprings (Schnupf *et al.*, 2013; Ericsson *et al.*, 2014 for review). Intracellular offsprings once formed are unicellular until they start to filament and become bicellular, then tricellular.

SFB have garnered much interest because of their unique ability to educate the gut immune system and to induce a healthy state of physiological inflammation (Schnupf *et al.*, 2013; Ivanov *et al.*, 2009). SFB colonization leads to the maturation of the gut mucosal lymphoid tissue, induces a strong and broad IgA response, stimulates the T-cell compartment, upregulates intestinal innate defence mediators, and induce a striking increase in the small-intestinal Th17 responses (Schnupf *et al.*, 2013; Ivanov *et al.*, 2009; Gaboriau-Routhiau *et al.*, 2009). In addition, SFB colonization exerts an adjuvant effect on systemic responses and can thus exacerbate pathologies in mouse models of encephalitis and arthritis, while protecting genetically predisposed mice against the development of type I diabetes (Lee *et al.*, 2011; Wu *et al.*, 2010; Chappert *et al.*, 2013; Kriegel *et al.*, 2011; Yurkovetskiy *et al.*, 2013).

Unlike other commensals, the segmented filamentous bacterium intimately attaches to absorptive epithelial cells in the ileum and cells overlying Peyer's patches (Jepson *et al.*, 1993; Chase *et al.*, 1976). This colonization does not result in pathology; rather, it protects the host from pathogens (Ivanov *et al.*, 2009).

The genomes of several SFB strains are known in the art. Recent sequencing of the rat and mouse SFB genomes revealed the highly auxotrophic needs of SFB and placed SFB between obligate and facultative symbionts (Pamp *et al.*, 2012). These findings suggest that SFB obtain at least some of their nutritional requirements from their interaction with the host (Prakash *et al.*, 2011; Szczesnak *et al.*, 2011; Kuwahara *et al.*, 2011; Pamp *et al.*, 2012).

Yet, little is known about the segmented filamentous bacterium–host interaction that underlies the important immunostimulatory properties of the bacterium, because SFB have resisted *in vitro* culturing for more than 50 years.

Therefore, there is a need for an effective method of culturing SFB *in vitro*.

The inventors have grown mouse SFB outside their host in a segmented filamentous bacterium–host cell co-culturing system. The inventors have shown that in this system, single-celled SFB isolated from monocolonized mice undergo filamentation, segmentation, and differentiation to release viable infectious particles, the unicellular intracellular offspring, which can colonize mice to induce signature immune responses. The inventors have also observed in this co-culturing system the formation of SFB spores. *In vitro*, intracellular offspring can attach to mouse and human host cells and recruit actin. Further, SFB can potentially stimulate the upregulation of host innate defence genes, inflammatory cytokines, and chemokines.

Accordingly, the present invention provides an *in vitro* method of culturing a segmented filamentous bacterium strain, comprising co-culturing said segmented filamentous bacterium strain with a eukaryotic host cell, wherein the culture is performed at an O₂ level inferior to 5%, preferably inferior to 4%, in a rich tissue culture liquid medium containing bacterial medium components including iron.

As used herein a segmented filamentous bacterium strain refers to a *Candidatus arthromitus* or *Candidatus savagella* or *Arthromitus immunis* or *Arthromitus muris* strain, preferably a *Arthromitus muris* strain.

Advantageously, the method comprises the steps of:

- a) growing a eukaryotic host cell on a solid culture medium;
- b) immersing the eukaryotic host cells grown in step a) in a eukaryotic host cell-SFB liquid culture medium;
- c) challenging the cell culture of step b) with a SFB strain, preferably an intracellular SFB offspring;
- d) co-culturing the live eukaryotic host cell and the SFB strain in the liquid culture medium at an O₂ level inferior to 5%, preferably inferior to 4%; and optionally
- e) recovering the cultured SFB strain.

With regard to step a):

The eukaryotic host cell can be any vertebrate cell, preferably any mammalian cell, more preferably any human or murine cell (*e.g.*, a mouse cell).

In a preferred embodiment the eukaryotic host cell is an adherent cell *in vitro*. Advantageously, this adherent cell is able to differentiate and hence fares well under confluent conditions for extended periods of time *in vitro*.

In another preferred embodiment, the eukaryotic host cell is an epithelial cell. Advantageously, the eukaryotic host cell is a cell from the gastrointestinal tract, such as the colon or small intestine.

In another embodiment, the eukaryotic host cell is a cancer cell, preferably a carcinoma cell, more preferably an adenocarcinoma cell.

In a most preferred embodiment, the eukaryotic host cell is a mammalian gastrointestinal epithelial adherent cell or a mammalian adherent cancer cell, in particular a human or mouse colon epithelial or small intestine cell or a human or mouse adenocarcinoma cell.

5 In a particular embodiment, the eukaryotic host cell is selected from the group consisting of the human Caco-2, TC7 (which is a subclone of Caco-2) or HeLa cell lines or mouse mICcl2 or CMT93 cell lines, preferably the human Caco-2 or TC7 cell line.

Advantageously, the eukaryotic host cell is grown at an O₂ level comprised between 0% (anaerobic conditions) and 5%, preferably between 0.5 and 3%, more preferably between
10 1 and 2.5%.

The humidity and temperature conditions for growing the eukaryotic host cell can be determined by routine tests by a person skilled in the art.

The composition of the eukaryotic host cell culture medium can be determined by routine tests by a person skilled in the art. By way of example, the culture medium comprises
15 Dulbecco's Modified Eagle Medium (DMEM) or Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) advanced medium, inactivated fetal calf serum (FCS) and amino acids.

Advantageously, the eukaryotic host cell is grown until a cell confluence of at least 20%, and by order of increasing preference of at least 25%, 30%, 35%, 40%, 50%, 55%,
20 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, and more preferably of 100%, is obtained.

In a preferred embodiment, the eukaryotic host cell is grown until a cell monolayer is obtained.

In another preferred embodiment, the eukaryotic host cells grown in step a) are
25 plated on a plate, preferably on a tissue culture well or transwell, more preferably on a tissue culture transwell, prior to step b).

With regard to step b):

The eukaryotic host cell-SFB liquid culture medium is a medium suitable for culturing both the eukaryotic host cell and the SFB strain.

30 Advantageously, said liquid culture medium is a rich tissue culture medium (e.g., DMEM/F12 advanced medium) containing bacterial medium components including iron.

Said liquid culture medium can comprise brain-heart infusion and a yeast/peptone/casein amino-acid mixture. These bacterial medium complements are well known in the art.

35 Iron can be in the ferrous form (Fe²⁺ state) and/or ferric (Fe³⁺ state). By way of example, iron can be in the form of Fe²⁺/Fe³⁺, 3xFe²⁺, 3xFe³⁺ or hemin, preferably Fe²⁺/Fe³⁺, 3xFe²⁺ or 3xFe³⁺.

Advantageously, iron concentration is comprised between 0.015 and 0.05 mM, preferably between 0.02 and 0.04 mM.

In a preferred embodiment, said liquid culture medium comprises a medium for culturing the eukaryotic host cell, such as DMEM/F12 advanced medium, supplemented with fetal calf serum, non-essential amino acids (such as GlutamaxTM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), brain-heart infusion, a yeast/peptone/casein amino-acid mixture and iron.

Advantageously, said liquid culture medium further comprises sugars, retinoic acid and/or nucleotides.

With regard to step c):

The SFB strain can be a wild-type strain or a genetically modified strain. A genetically modified SFB strain can be obtained from a wild-type SFB strain by transformation, transduction or conjugation.

The SFB strain can be in the form of a filament or an intracellular offspring.

The SFB strain can be isolated from a subject, in particular a mammal, such as a human or a mouse (*e.g.*, a SFB monoassociated mouse). Methods for isolating a SFB strain from a mammal are known in the art (Lécuyer *et al.*, 2014).

A SFB filament can be isolated by the following method: the ileal, caecal, and/or colonic contents from a subject is collected and the individual filament is captured using an inverted microscope and a micropipette attached to a micromanipulator.

An intracellular SFB offspring can be isolated under anaerobic conditions by the following method: the ileal, caecal, and/or colonic contents from a subject is collected, filtered through a 100- μ m mesh, separated from other faecal matter using a density gradient column and passed through a 5- μ m filter to obtain a pure culture of unicellular intracellular offspring (average 0.7 μ m). SFB filaments can also be collected on 5 μ m filters following this method.

As used herein, the term “challenging” refers to the addition of a bacterium to an eukaryotic host cell regardless of whether this bacterium interacts (attaches/invades) or not the host cell.

In a preferred embodiment, the SFB strain is closely contacted with the eukaryotic host cell, preferably is in direct contact with the eukaryotic host cell.

As used herein, the term “closely contacted” refers to a co-culture of a eukaryotic host cell and a SFB strain wherein the distance between said eukaryotic host cell and said SFB strain is inferior or equal to 2 cm.

With regard to step d):

The co-culture of the eukaryotic host cell and the SFB is performed at an O₂ level comprised between 0% (anaerobic conditions) and 5%, preferably between 0.5 and 3%, more preferably between 1 and 2.5%.

The humidity and temperature conditions for co-culturing the live eukaryotic host cell and the SFB strain can be determined by routine tests by a person skilled in the art.

The co-culture can be performed for 1, 2, 3, 4, 5 or 6 days.

The co-culture can also be performed until the SFB strain undergoes filamentation, segmentation, differentiation to release intracellular offsprings or spores.

Conditions to induce spore formation advantageously include adding a stress such as oxidative stress or short times of antibiotics (Davis and Savage, 1976).

With regard to step e)

The cultured SFB strain can be recovered in a form of a filament, an intracellular offspring or a spore.

A SFB filament can be recovered using an inverted microscope and a micropipette attached to a micromanipulator.

A SFB intracellular offspring can be recovered from a filament by filtration through a 5- μ m filter. SFB filaments can be collected on 5 μ m filters.

A SFB spore (that is a bacterial spore) can be recovered by a method well known in the art, such as the methods disclosed in International Application WO 2014/121298.

The present invention also provides the *Arthromitus muris* strain deposited by the Applicant, according to the Budapest Treaty, at CNCM (Collection Nationale de Cultures de Microorganismes, 25 rue du Docteur Roux, Paris) on December 23, 2014, under the accession number CNCM I-4932.

The present invention also provides a method for genetically modifying a segmented filamentous bacterium strain by conjugation, comprising the steps of:

i) genetically modifying an *Escherichia coli* strain with a recombinant shuttle vector DNA comprising a DNA sequence of interest to introduce in the SFB strain, wherein the said shuttle vector DNA is capable of replicating in the *E. coli* strain, and in the SFB strain to be targeted for genetic modification;

ii) co-culturing the genetically modified *E. coli* strain obtained in step i) together with the SFB strain to be targeted for genetic modification according to the *in vitro* method of culturing of the invention, wherein:

in step c) of said method of culturing of the invention, the cell culture of step b) is challenged with both the SFB strain to be targeted for genetic modification, preferably an intracellular SFB offspring, and said genetically modified *E. coli* strain ; and

in step d) of said method of culturing of the invention, the live eukaryotic host cell, the SFB strain and the genetically modified *E. coli* strain are co-cultured in the liquid culture medium at an O₂ level inferior to 5%;

iii) recovering the genetically modified SFB strain of step ii).

The recombinant shuttle vector DNA can be a recombinant Clostridial shuttle vector DNA comprising a DNA sequence of interest and capable of replicating both in an *E. coli*

strain and in a Clostridia strain. The construction of such a vector is known in the art (Heap *et al.*, 2009).

Advantageously, the DNA sequence of interest can be under the control of a constitutive SFB, such as ribosomal, promoter for cytosolic expression or fused to a nucleotide sequence encoding a secreted lipoprotein for surface expression.

The recombinant shuttle vector DNA can comprise different selectable antibiotic markers.

The DNA sequence of interest can be a DNA sequence encoding an antigen of interest. By way of example, the antigen of interest can be IpaB or IpaD from *Shigella flexneri*.

The *E. coli* strain can be a negatively-selectable auxotrophic *E. coli* strain (Danchin, 1977).

Advantageously, the *E. coli* strain requires diaminopimelic acid to grow. Such a strain can be selected against diaminopimelic acid in a rich culture medium.

The genetic modification of the *E. coli* strain can be carried out by electroporation, transduction, heat shock transformation or protoplast fusion.

The co-culturing of step ii) allows transferring the said shuttle vector DNA from the *E. coli* strain to the SFB by conjugation.

Advantageously, the cell culture of step b) is challenged first with the SFB strain to be targeted for genetic modification, and then with said genetically modified *E. coli* strain after there is outgrowth of the SFB strain.

The present invention also provides a method for genetically modifying a segmented filamentous bacterium strain by electroporation, comprising the steps of:

i) culturing and recovering a segmented filamentous bacterium strain according to the *in vitro* method of the invention;

ii) mixing the SFB strain recovered in step i) with a purified plasmid to be transformed, preferably in glycerol/H₂O (*e.g.*, 10% glycerol/H₂O);

iii) applying an electric pulse to the mixture obtained in step ii);

iv) recovering the genetically transformed SFB strain.

In step i) of this method, the SFB strain can be cultured for 2-4 days on TC7 cells.

In step i) of this method, the SFB strain can be recovered by collecting the SFB cell and putting it on ice under O₂ level comprised between 0% (anaerobic condition) and 5%, preferably between 0.5 and 3%, more preferably between 1 and 2.5%.

In step ii) of this method, the electric field is advantageously comprised between 6 kV.cm⁻¹ and 30 kV.cm⁻¹, preferably between 6.5 kV.cm⁻¹ and 25 kV.cm⁻¹.

In step ii) the purified plasmid can be designed for use in Clostridia species (Heap *et al.*, 2009).

The present invention also provides a method for genetically modifying a segmented filamentous bacterium strain by chemical transformation, comprising the steps of:

i) culturing and recovering a segmented filamentous bacterium strain according to the *in vitro* method of the invention;

5 ii) mixing the SFB strain recovered in step i) with a purified plasmid to be transformed, preferably in glycerol/calcium chloride (*e.g.*, 10% glycerol/0.1M calcium chloride);

iii) incubating the mixture obtained in step ii);

iv) optionally applying a heat shock to the mixture;

10 v) recovering the genetically transformed SFB strain.

In step i) of this method, the SFB strain can be cultured for 2-4 days on TC7 cells.

In step i) of this method, the SFB strain can be recovered by collecting the SFB cell and putting it on ice under O₂ level comprised between 0% (anaerobic condition) and 5%, preferably between 0.5 and 3%, more preferably between 1 and 2.5%.

15 In step iii), the SFB strain and the purified plasmid can be incubated for 15 to 45 min, preferably 30 min.

In step iv), the heat shocked is advantageously applied at 41-43 °C, preferably at 42°C for 15 to 45 seconds, preferably 30 seconds.

20 A live genetically modified SFB strain can be used as an antigen delivery vehicle, either as filament, intracellular offspring or spore, preferably as a spore, after oral ingestion or parenteral administration to a subject, preferably a mammal.

Accordingly, the present invention also provides an isolated genetically modified SFB strain expressing an antigen of interest (*e.g.*, IpaB or IpaD from *Shigella flexneri*), preferably secreting said antigen or exposing said antigen on bacterium's surface.

25 As used herein, an antigen of interest refers to an antigen derived from a microorganism or a cell (*e.g.*, tumor cell) against which one intends to elicit an immune response in a subject.

30 The genetically modified SFB strain can be obtained by transformation, transduction or conjugation as described above, preferably by a method for genetically modifying a segmented filamentous bacterium strain according to the invention.

The present invention also provides a live genetically modified SFB strain expressing an antigen of interest as defined above for use as a medicament, in particular for use in preventing or treating a disease caused by a microorganism from which said antigen derives, or a cancer.

35 Said SFB strain can be in the form of a filament, an intracellular offspring or a spore, preferably a spore.

The present invention also provides a therapeutic immunogenic (or vaccine) composition comprising a live genetically modified SFB strain expressing an antigen of interest as defined above.

5 Said SFB strain can be in the form of a filament, an intracellular offspring or a spore, preferably a spore.

The terms “immunogenic composition” and “vaccine composition” are used interchangeably herein.

10 The present invention also provides an immunogenic composition as described above for use in preventing or treating a disease caused by a microorganism from which said antigen derives or a cancer.

The genetically modified SFB strain expressing an antigen of interest and the immunogenic composition comprising the said genetically modified SFB strain according to the present invention are particularly suitable for eliciting in a subject antibodies directed to said antigen.

15 Advantageous said immunogenic composition comprises a pharmaceutically acceptable carrier.

As used herein, a “pharmaceutically acceptable carrier” includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration.
20 Suitable carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field.

The genetically modified SFB strain expressing an antigen of interest and the immunogenic composition comprising the said genetically modified SFB strain according to the present invention are administered to a mammal subject, preferably a human, in an
25 amount sufficient to prevent or attenuate the severity, extent of duration of the infection by the microorganism from which said antigen derives or a cancer.

The therapeutically effective amount varies depending on the subject being treated, the age and general condition of the subject being treated, the capacity of the subject's immune response to synthesize antibodies, the degree of protection desired, the severity of
30 the condition to be treated, and its mode of administration, among other factors. An appropriate effective amount can be readily determined by one of skill in the art. A therapeutically effective amount will fall in a relatively broad range that can be determined through routine trials.

Following an initial vaccination, the subject may receive one or two booster
35 injections at an appropriate interval determined by one of skill in the art.

Typically, the immunogenic composition is prepared as an injectable form (either a liquid solution or suspension) or as a solid form suitable for solution or suspension in a liquid carrier prior to injection.

Once formulated, the immunogenic composition may be administered parenterally or by mucosal routes, preferably orally.

The present invention also provides the use of a genetically modified SFB strain expressing an antigen of interest or an immunogenic composition as defined above for the manufacture of a medicament, preferably of a preventive or therapeutic vaccine against a microorganism from which said antigen derives in a subject or a cancer.

The present invention also provides a method for preventing and/or treating a infection caused by a microorganism from which said antigen derives or a cancer, comprising administering to a subject in need thereof a genetically modified SFB strain expressing an antigen of interest or an immunogenic composition as defined above, in an amount effective to inhibit said microorganism infection of susceptible cells so as to thereby prevent or treat the infection or to inhibit the growth of cancer cells so as to thereby prevent or treat cancer.

The term “treating” includes the administration of a genetically modified SFB strain expressing an antigen of interest or an immunogenic composition as defined above to a patient who has an infection, a symptom of infection or a cancer, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the infection and/or the symptoms of the infection, or the cancer.

The term “preventing” means that the progression of an infection is reduced and/or eliminated, or that the onset of an infection or cancer is delayed or eliminated.

The present invention also provides the use of a SFB strain obtainable by the *in vitro* method of culturing of the invention, as a probiotic.

Said SFB strain can be alive or dead, preferably alive.

Said SFB strain can be in the form of a filament, an intracellular offspring or a spore, preferably a spore.

The present invention also provides a food product (*e.g.*, dairy product) comprising a SFB strain obtainable by the *in vitro* method of culturing of the invention.

Said SFB strain can be alive or dead, preferably alive.

Said SFB strain can be in the form of a filament, an intracellular offspring or a spore, preferably a spore.

In addition to the preceding features, the invention further comprises other features which will emerge from the following description, which refers to examples illustrating the present invention, as well as to the appended figures.

Figure 1: Growth and growth requirements of SFB *in vitro*. **a**, Gram stain and length of SFB before and after 5- μ m filtration; **b**, qPCR of SFB growth on host cells at the indicated conditions; **c**, SEM images of 4-day-old SFB filaments grown anaerobically on TC7 cells on transwells; **d–i**, SFB growth at low oxygen concentrations (**d**), on the indicated cell line seeded on transwells; **e**, on TC7 cells at indicated cell confluence; **f**, **g**, under various

b, i, h, Statistical analysis using the two-tailed *t*-test (**P* = < 0.05, ***P* = < 0.01, ****P* = < 0.001).

Figure 2: Differentiation of SFB from filaments to intracellular offspring during *in vitro* growth. **A**, Schematic representation of an SFB filament highlighting stages of its growth and differentiation. Gram stain of SFB after 4 days of growth on **B**, TC7 cells incubated at the indicated condition, and **C**, TC7 cells grown on transwells in low oxygen. Analysis of SFB grown on mICcl2 cells (**D–G**) and TC7 cells (**H**) on transwells at 1–2.5% O₂: **D**, qPCR quantification (mean ± s.d. of triplicates); **E**, length of individual SFBs; **F**, Gram stain; and **G**, segment length analysis of representative 2-day-old SFB filaments. **H**, SEM of SFB after 4 days of growth. **B–H**, Representative images and values from one of three experiments performed in triplicate with (**D**) means ± s.d.

Figure 3: Viability, colonization, and immunostimulatory potential of *in vitro*-grown SFB. **a**, SFB length after growth on TC7 cells on transwells; **b**, quantification and **c**, Gram stain of SFB growth on TC7 cells on transwells before and after a 3-day sub-culturing of the 5-µm filtrate; **d–k**, analysis of germ-free C57BL/6 mice gavaged with either filament/intracellular offspring mix, intracellular offspring, or faeces of SFB-monoassociated mice (SFB*Vivo*); **d**, quantification of ileum-associated SFB; **e, k**, Host gene expression in the ileal lamina propria; representative flow cytometry plots and quantification of (**f, g**) B220+ B cell and (**i, j**) CD45+CD3+CD4+ T cell frequencies of the indicated markers; **h**, faecal secretory IgA quantification by ELISA. **a–k**, Images and values are representatives from one of two experiments performed (**a–c**) in duplicate with means ± s.d. or (**d–k**) performed with four to six mice per group. Statistical analysis using the two-tailed *t*-test (****P* = < 0.001).

Figure 4: SFB–host cell interaction and host response. SEM of SFB attached to subconfluent (**A**) and confluent (**B**) mICcl2 cells after 2 days of intracellular offspring challenge. **C**, Merge (**M**) of 4',6-diamidino-2-phenylindole (DAPI) (**D**) and actin (**A**) stain of mICcl2 cells challenged with intracellular offspring for 2 days showing confocal slices (**s**) and Z-projections. **D**, SEM of 3-day-old SFB filament attached to TC7 cells. Host response using qPCR analysis of mICcl2 cells (**E, F, H**) and TC7 cells (**G**) 3 days after challenge with (**E–G**) SFB or (**H**) various MAMPs. NS, not significant; BDL, below detection limit. Images and values are representatives from one of two experiments performed in triplicate (**A–D**) or are cumulative values from four experiments performed in triplicate with box plots of 25–75% centiles with min/max whiskers (**E–G**) or means ± s.e.m.; (**H**) with a two-tailed *t*-test statistical analysis (**P* = < 0.05, ***P* = < 0.01, ****P* = < 0.001).

Figure 5. Intestinal colonization of *in vitro*-grown SFB and host response. **a, b**, qPCR quantification (**a**) and SEM (**b**) of *in vitro*-grown SFB used for gavage. **c**, qPCR quantification of SFB in faecal samples. **d**, SEM of SFB attachment *in vivo* at 25 days after gavage of C57BL/6 mice with SFB*Vivo* or SFB*Vitro*. **e**, SEM of 21-day SFBcolonized germ-free C3H/HeN mouse ileum showing vacant attachment sites. **f**, Host gene expression in the

ileal lamina propria in conventional or germ-free mice colonized with either SFB or *E. coli* for 21 days. Images and values are representatives from one of two experiments (a–e) or are cumulative values from two experiments performed with a total of seven germ free, four SFB, five *E. coli*, and four conventional mice (f), showing box plots of 25–75% centiles with median and minimum/maximum whiskers. c, f, Two-tailed *t*-test statistical analysis (**P* = < 0.05, ***P* = < 0.01).

EXAMPLE 1: GROWTH AND HOST INTERACTION OF MOUSE SEGMENTED FILAMENTOUS BACTERIA IN VITRO

Materials and Methods

Cell culture and SFB-specific culture medium

TC7, CMT93, and HeLa cells were cultured in DMEM (Gibco 31885) with 10% inactivated fetal calf serum (FCS; AbCys CVFSVF00-0U) and non-essential amino acids (Invitrogen 11140-035). mICcl2 were maintained in DMEM/F12 advanced medium (Gibco 12634) with 2% inactivated FCS and Glutamax (Gibco 35050) and supplemented with 10 nM hEGF (Sigma E9644), 50 nM dexamethasone (Sigma D4902) and 1 nM triiodothyronine (Sigma T5516). HeLa cells were obtained from ATCC. All cell lines were tested for mycoplasma every 2 weeks and always found to be negative. Cells were plated either on regular 12-well tissue culture plates or on Costar transwell plates with 0.4-μm filters (Sigma CLS3460 and Fisher Scientific W2127P) from 1 to 3 days before the experiment such that a monolayer was present at the start of the experiment and added to a low oxygen chamber overnight for slowly lowering oxygen concentrations in the medium of the cells. At the start of SFB culturing, the TC7, CMT93, HeLa and mICcl2 medium is first replaced with (equilibrated: 1% oxygen) medium before SFB addition (directly to the TC7 cells).

SFB medium was made up as follows: DMEM/F12 advanced medium with 2% FCS, Glutamax and 12.5 mM HEPES (Sigma H0887) with the following supplementation: 1 in 100 dilution of (1) brain–heart infusion (BD Difco 237500) 5× concentrated, (2) peptone/yeast (BBL Biosafe 211862) at 10% and casein amino acids at 5% (DIFCO 0320-01-1), (3) ribose/cellobiose/mannose (Sigma: R9629, C7252, M6020) at 200 mM; 1 in 1,000 dilution of (1) ferrous sulphate (Merck 3965) at 10 mM, (2) ferric ammonium citrate (LabGuard 0658) at 12.5 mM, (3) Hemin (Sigma 51280) at 1.5 mM in 50% ethanol with 1.4 N NH₄OH, (4) sodium ascorbate at 10 mg ml⁻¹ with 1-phosphoascorbate at 500 mM (Sigma: A4034, 49752); 1 in 10,000 dilution of retinoic acid (Sigma R2625) at 30 mg ml⁻¹ in DMSO; 1 in 500 dilution of (1) sperm DNA (Life Technologies 15632-011) at 10 mg ml⁻¹ digested for 1 h with 10 μl DNaseI (Roche 04 716 728 001) at 37 °C and heat inactivated at 75 °C for 30 min, (2) RNA at 10 mg ml⁻¹ (Sigma R6750) undigested. SFB medium specific medium supplements, except for the nucleotides and hemin were prepared fresh every 2 weeks and otherwise stored at 4 or –20 °C for retinoic acid, and nucleotides. When mICcl2 cells were used, SFB medium was further supplemented with hEGF, dexamethasone, and

triiodothyronine. Notably, additional supplementation with 0.2% yeast extract (BD 212750; 1/100 from 20%) further improves SFB growth and it is not necessary to add ascorbate, phosphoascorbate or hemin.

Purification of intracellular offspring from SFB-monoassociated mice and infection protocol

All liquids used for the isolation of intracellular offspring were pre-equilibrated overnight in an anaerobic chamber set to 0% oxygen. SFB-monoassociated JH^{-/-} mice were killed aseptically in a tissue culture hood and then placed in an anaerobic cabinet for dissection. The ileal, caecal, and colonic contents were resuspended in 50 ml PBS and homogenized by vortexing. Homogenates were passed through a 100- μ m mesh to remove large faecal debris. The filtrate was spun at 8,000g for 5 min to pellet bacteria and insoluble material, and the pellet was resuspended in 3 ml PBS per mouse killed, layered onto 3 ml 50% and 2 ml 30% Nycodenz (AbCYS 1002424) solution made with PBS in 15 ml Falcon tubes and spun for 10 min at 4,000g. SFB within the 30% fraction were collected, diluted in PBS, and bacteria were pelleted for 10 min at 8,500g. Pellets were resuspended in 15 ml pre-equilibrated PBS by pipetting/vortexing and filtered through a 5- μ m filter (Sigma Z612502). The filtrate was again centrifuged for 5 min at 8,000g and the pellet was resuspended in an appropriate amount of pre-equilibrated culture medium. Usually one mouse was killed for every four 12-well plates used, and 50 μ l of bacterial suspension was added to each well. To facilitate adhesion, cells challenged with SFB were sealed in ziplock bags within the cabinets, removed from the cabinet, and spun for 10 min at 300g.

SFB are allowed to grow in the presence of eukaryotic cells for 3 days during which the oxygen concentration is increased incrementally from 1% on day 0 to 1.5% on day 1, to 2% on day 2, and to 2.5% on day 3. Subcultures can be prepared on day 3 by taking aliquots of the culture supernatant and using this to challenge freshly prepared eukaryotic cells (prepared as described above on transwell filters and at low oxygen conditions).

SFB recovery, and quantification and analysis of SFB growth

To recover SFB, the culture supernatant was collected and centrifuged for 4 min at 8,000g and the pellet was resuspended in 100 μ l PBS, of which 20 μ l was spotted on glass slides for the Gram stain, 30 μ l was mixed with an equal volume of 50% glycerol and frozen at -80 °C, and the remaining 50 μ l was used for DNA extraction. The DNA was isolated with a Qiagen stool kit (51504; without the use of the inhibitor tablet) and diluted 1 in 20. SFB growth was enumerated by qPCR analysis of the 16S rRNA genes using the following primer pairs: the SFB specific F: 5'-AGGAGGAGTCTGCGGCACATTAGC-3' (SEQ ID NO: 1); and the universal R: 5'-TCCCCACTGCTGCCTCCCGTAG-3' (SEQ ID NO: 2). For qPCR, 6 μ l of diluted SFB DNA was mixed with 1.5 μ l of a 4 mM primer mix and 7.5 μ l of Power SybrGreen Master mix (Applied 4368708) and run on an ABI 7900HT machine in a 384-well plate. Statistical analysis used a two-tailed Student's *t*-test (**P* = < 0.05, ***P* = < 0.01,

*** $P = < 0.001$). SFB segment length analysis used ImageJ. For SEM analysis, SFB-containing supernatants were washed with PBS and suctioned onto 0.1- μm filters (Watman 110405) and fixed in 0.1 M cacodylate buffer containing 2.5% glutaraldehyde before being processed. Cells for SEM were fixed in PHEM buffer (18.14 g PIPES, 6.5 g HEPES, 3.8 g EGTA, 0.99 g MgSO_4 per litre with 10 M KOH to pH 7.0) containing 4% sucrose and 2.5% glutaraldehyde, and processed for SEM. For fluorescence, cells were fixed in PBS/3.7% PFA, permeabilized with PBS/0.1% Triton X-100, stained with DAPI and A568-phalloidin, and stacks of 0.4- μm slices were taken on a Leica SP5 confocal microscope.

Colonization of germ-free mice with *in vitro*-grown SFB

This experiment was done independently twice with similar results; one experiment is shown. SFB grown *in vitro* for 3 days on TC7 and mICcl2 cells on transwells were divided in equal parts and one half was filtered through a 5- μm filter to obtain a fraction containing intracellular offspring only. Bacteria were concentrated by centrifugation to obtain 0.25 ml of bacteria in PBS per mouse. The number of animals used followed availability of animals, isolators, and input quantities. Randomization or blinding was otherwise not performed. Two groups of four 11-week-old C57BL/6 male and female mice maintained at the germ-free facility were starved for one night, gavaged with 0.25 ml 400 mM sodium bicarbonate, followed by 0.25 ml of *in vitro*-grown SFB. Age-matched control mice were colonized with *in vivo*-derived SFB as described below. Faecal samples were collected for each mouse at various times during a 3 week period, SFB DNA was extracted using a Qiagen stool kit and quantified on the basis of qPCR analysis of 16S rDNA and comparison with a SFB DNA sample of known SFB genome concentration as determined by Illumina sequencing. To monitor SFB associated with the ileum, DNA was extracted from frozen ileal biopsies using the method in Godon *et al.*, 1997.

Isolation and staining of lamina propria lymphocytes from C57BL/6 mice

Age-matched germ-free B6 mice and mice colonized with either *in vitro*- or *in vivo*-grown SFB for 3 weeks were analysed for their innate and adaptive immune response as described in Ivanov *et al.*, 2009. Briefly, after excision of Peyer's patches, the mouse small intestine was washed in PBS, and ileal samples were placed in RNAlater for RNA extraction, complementary DNA (cDNA) synthesis, and qPCR analysis using SYBR or Taqman technologies (Applied Biosystems) and a QuantStudio7 qPCR machine. Values were normalized to TfrC.

Lamina propria lymphocytes (LPL) were prepared as previously described by Gaboriau-Routhiau *et al.*, 2009. The remaining small intestine was incubated four times in 60 ml of PBS-3 mM EDTA (Sigma) for 10 min at 37 °C, and digested in 60 ml of RPMI 1640 with 20% FCS (Gibco), 100 U ml^{-1} collagenase (Sigma), and 175 U ml^{-1} DNase I (Sigma) for 40 min at 37 °C. LPL were then purified on a 40–80% Percoll gradient run for

15 min at 2,000g and resuspended in DMEMGlutamax with 8% FCS, 1 mM HEPES, 0.02 mM folic acid, 0.67 mM L-arginine, and 0.27 mM L-asparagine (all from Sigma).

Analysis of LPL for surface antigens and intracellular expression of IL-17 and IL-10 was by flow cytometry as described⁵. Briefly, LPL were stimulated for 4 h with 100 ng ml⁻¹ phorbol 12-myristate 13-acetate and 1 µg ml⁻¹ ionomycin, in the presence of Brefeldin A (10 µg ml⁻¹) (all from Sigma). Cells used for surface analysis were left unstimulated. For surface staining, LPL were labelled for 20 min at 4 °C with a cocktail of the following antibodies: FITC-anti-GL7 (clone GL7), PerCP-anti-CD8a (clone 53-6.7), APC-H7-anti CD4 (clone GK1.5), AF647-anti-B220 (clone RA3-6B/2) (all from BD Pharmingen), PE-anti-IgA (Southern Biotech), and eFluor450-anti-CD45 (clone 30-F11) and PECy7-anti-CD3 (clone 145-2C11) (both from eBioscience).

For intracellular cytokine staining, cells were further fixed in 2% PFA for 20 min at room temperature (~24 °C), and washed and stained overnight at 4 °C with PE-anti-IL-17 (clone TC11-18H10) and APC-anti-IL-10 (clone JES5-16E3) (BD Pharmingen) diluted in PBS-1% FCS-0.5% saponin (Sigma). Labelled cells were analysed with a FACSCanto II and FACSDiva software (BD Biosciences). Gates were set on living cells after Aqua live/dead dye exclusion (Invitrogen).

For qPCR analysis, the median value of germ-free mice was calculated and used as the reference value of 1 for comparison of the median value of the test samples.

20 Colonization of germ-free mice with SFB from faeces and *E. coli*

Germ-free male and female C3H/HeN mice were obtained from INRA (ANAXEM platform) germ-free facilities. Eight- to nine-week-old germ-free mice were gavaged with 0.5 ml of either fresh anaerobic cultures of *E. coli* MG1655 or faecal homogenate from SFBmonoassociated mice (Ivanov *et al.*, 2009). Colonization by SFB was monitored in faeces through bacterial DNA extraction and 16S rDNA amplification by qPCR using specific primer pairs for SFB. Values were normalized to Ccl25, a constitutively expressed epithelial cell marker, and compared with the median value from germ-free control mice. Germ-free and gnotobiotic mice were maintained in plastic isolators and fed *ad libitum* on a commercial diet sterilized by γ-irradiation (40 kGy). The numbers of animals used followed availability of animals, isolators, and were obtained from two independent experiments. Randomization or blinding was otherwise not performed. Gnotobiotic mice were killed on day 21 after colonization in parallel with age-matched germ-free controls. All animal procedures were performed in accordance with French legislation and EEC regulations for the care and use of laboratory animals, approved by the local ethics committee and authorized by the French Ministry of Research.

35 Host response in vitro to SFB growth and MAMP stimulation

Host response to SFB and MAMPs included pooled results from four independent experiments with three technical samples. Generally a minimum of triplicate biological

replicates were used and increased if trends were clear but significance not. After 3 days of *in vitro* growth of SFB on either mICcl2 or TC7 cells on transwells in SFB medium lacking hemin and sodium ascorbate, at 1–2.5% oxygen, cells were lysed and RNA was extracted using a Nucleospin RNA kit (Macherey-Nagel). cDNA was synthesized using RNA superscript II, oligo dT, RNaseout, and dNTPs (Invitrogen), and pPCR was performed on an ABI 7900HT and QuantStudio7 (Life Technologies) qPCR machine using the protocol described by Schnupf *et al.*, 2012. TaqMan assays were performed as suggested by the supplier. Values were normalized to B2M and Ct values for Reg3 γ , Tnf α , and Fabp2 were set to 41 in control cells owing to the lack of transcript detection. MAMP stimulation used the following agonists at the highest concentrations recommended by the supplier (Invivogen): Pam2CSK4 (tlrlpm2 s-1) at 100 ng ml⁻¹, Pam3CSK4 (tlrl-pms) at 300 ng ml⁻¹, peptidoglycan of *E. coli* K12 (Tlr-ksspgn) at 10 μ g ml⁻¹, MDP (tlrl-mdp) at 10 μ g ml⁻¹, CpG (tlr-1584) at 3 μ g ml⁻¹, flagellin (tlrl-pstfla-5) at 100 ng ml⁻¹. Excess for flagellin (10 $\times$) was also tested and found to be similar.

Mouse and human qPCR primers used

Mouse Taqman® assays:

B2M Mm00437762_m1, TfrC Mm00441941_m1, Ccl25 Mm00436443_m1, Fabp2 Mm00433188_m1, Reg3g Mm01181783_g1, Fut2 Mm00490152_S1, Aqp3 Mm01208559_m1, Pigr Mm00465049_m1, Ltf Mm00434787_m1, Tff3 Mm00495590_m1, Tnfa Mm00443258_m1, IL1a Mm00439620_m1, IL1b Mm00434228_m1, IL6 Mm00446190_m1, IL15 Mm00434210_m1, IL18 Mm00434225_m1, Cxcl2 Mm00436450_m1, Ccl2 Mm00436450_m1, Ccl5 Mm01302428_m1, Ccl7 Mm01308393_g1, Ccl20 Mm01268754_m1, Ccl28 Mm00445039_m1, Csf2 Mm01290062_m1

Mouse Sybr® primers

B2M: F:tcagtcgtcagcatggctcgc (SEQ ID NO: 3); R:tccggtgggtggcgtgagtatac (SEQ ID NO: 4)
iNos: F:cagctgggctgtacaaacctt (SEQ ID NO: 5); R:cattggaagtgaagcgtttcg (SEQ ID NO: 6)
Saa1: F:cattgtgtcacgaggtttcc (SEQ ID NO: 7); R:gttttccagttagcttcctcatgt (SEQ ID NO: 8)
Saa2: F:tgtgtatcccacaaggtttcaga (SEQ ID NO: 9); R:ttattaccctctcctcctcaagca (SEQ ID NO: 10)
Saa3: F:cgcagcacgagcaggat (SEQ ID NO: 11); R:ccaggatcaagatgcaaagaatg (SEQ ID NO: 12)
Cxcl1: F:tggtgtgggattcacctcaag (SEQ ID NO: 13); R:caagcctcgcgaccattct (SEQ ID NO: 14)
Cxcl10: F:gccgtcattttctgcctcat (SEQ ID NO: 15); R:gcttcctatgccctcatt (SEQ ID NO: 16)

Human Taqman® assays:

B2M Hs00984230_m1, Saa1 Hs00761940_s1, Saa2 Hs01667582_m1, IL6 Hs00985639_m1, IL8 Hs99999034_m1, Ccl20 Hs01011368_m1, Reg3g Hs00417999_m1, iNos Hs01075529_m1, Cxcl10 Hs00171042_m1, Fut2 Hs00382834_m1, IL15 Hs01003716_m1, IL18 Hs01038788_m1, Ccl5 Hs00982282_m1

Results

SFB–host cell co-culturing system

SFB strain (Bolotin *et al.*, 2014) isolated from monoassociated mice was deposited at CNCM (Collection Nationale de Cultures de Microorganismes, 25 rue du Docteur Roux, Paris) on December 23, 2014, under the accession number CNCM I-4932 (*Arthromitus muris* strain referred to as SFB-mouse-NL). SFB were cultured with eukaryotic cells grown in low but physiological oxygen conditions (He *et al.*, 1999) in a rich tissue culture medium containing bacterial medium components and additional supplements. SFB from monoassociated mice were collected, filtered through a 100- μ m mesh, separated from most other faecal matter using a Nycodenz column and passed through a 5- μ m filter to obtain a pure culture of unicellular intracellular offspring (average 0.7 μ m) (Fig. 1a). Eukaryotic cells grown on tissue culture wells or transwells were placed in a humidified anaerobic cabinet, challenged with intracellular offspring, and kept at either strict anaerobic conditions within a sealed box or left in the anaerobic cabinet where the oxygen concentration was maintained at low levels (0.5–1.4% O₂). After 4 days, bacterial growth in the culture supernatant was quantified by quantitative PCR (qPCR) using SFB-specific 16S rDNA primers (Fig. 1b) and confirmed using scanning electron microscopy (SEM) (Fig. 1c). SFB growth was observed in all conditions for most cell lines assayed but growth was usually enhanced on transwells compared with tissue culture wells and growth was significantly better in the presence of oxygen, revealing SFB to be a relatively aerotolerant anaerobe. Both human (TC7/HeLa) and mouse (mICcl2/CMT93) cell lines supported growth but the TC7 was the most resilient cell line generally supporting SFB growth most robustly. On the basis of temporal analysis, the highest exponential growth phase occurred between days 1 and 3, with an average maximum doubling time of 5.0 h (Fig. 1d).

Growth requirements for SFB

SFB growth had a striking dependence on host cell number, decreasing in number with decreasing cell density (Fig. 1e). In addition, negligible growth occurred in medium alone, medium supplemented with cell lysate, or when cells were fixed before SFB challenge (Fig. 1f), indicating that live host cells are required for SFB proliferation. SFB also required close contact for efficient growth as only little growth (0–6%) occurred when intracellular offspring were added to the bottom chamber of transwells or when bacteria were placed in transwells above TC7 cells in tissue culture wells (Fig. 1f, g). Yet, as host cell contact was not an absolute requirement, it suggests that host cells may release a soluble factor that promotes SFB growth. To address the requirement for medium supplementation, SFB were grown on TC7 cells in complete medium or medium missing individual additives. Brain–heart infusion, a yeast/peptone/casein amino-acid mixture, and particularly iron supplementation were critical for SFB growth (Fig. 1h, i).

In the 1970s, transmission electron microscopy studies of SFB present in the murine gut led to a proposed life cycle: attachment to epithelial cells via the holdfast at the intracellular offspring tip leads to filamentous growth and is followed by a complex developmental progression that starts at the distal tip and ultimately leads to intracellular offspring formation and release (Fig. 2A) (Chase *et al.*, 1976; Ferguson *et al.*, 1979). According to this model, when filaments grow beyond 50 μm in length, the large primary filament segments start to undergo a symmetrical division to form smaller secondary segments. These differentiate by dividing asymmetrically to form a mother/daughter cell. The daughter cell becomes engulfed and subsequently divides to form two intracellular offspring within the surrounding mother cell segment. Intracellular offspring are then released from the filament by breakdown of the filament septa and cell wall and reattach to the host.

In vitro, growth of SFB on TC7 cells often yielded considerable quantities of long filaments that could be clumped together in a hairball-like phenotype easily seen with the naked eye (Fig. 2B). Most of these filaments were undifferentiated after 4 days, with only some filaments showing a characteristic heterogeneous staining of differentiating filaments (Fig. 2B, left-hand column, bottom two panels). Intracellular offspring could be seen located at the filament tip or occasionally in the central part of a filament (Fig. 2C), similar to filaments recovered from SFB-monoassociated mice (data not shown). Differentiation of SFB occurred on all four cell lines tested, but we noticed that differentiation was more pronounced when higher oxygen concentrations (1–2.5%) were used. In time-course analysis at this higher oxygen concentration (Fig. 2D), only short bacteria were detected 1 day after challenge (Fig. 2E, F). After 2 days, only long filaments were present (Fig. 2E, F) and the bacterial septa could be identified quite clearly using the Gram stain (Fig. 2F). Three types of filament were found (Fig. 2F, G): short filaments with long intracellular segments of $\sim 2.6 \mu\text{m}$; medium-sized filaments with smaller intracellular segments of $\sim 1.2 \mu\text{m}$; and medium to long filaments that had a more heterogeneous distribution of segment lengths including very small segments (Fig. 2F, G) and rare segments with half-circular structures (Fig. 2F, Day 2-3, arrow) that resembled the engulfment of a daughter cell by a mother cell. After 3 days of growth, most filaments had differentiated, at least in part, to the final intracellular offspring stage and intracellular offspring could be seen in a characteristic doublet orientation at the filament tip where the filament cell wall appeared to have lost its structure (Fig. 2F, far left column). Many intracellular offspring of varying lengths were also no longer associated with filaments. SEM confirmed the presence of the needle-like holdfast structure on intracellular offspring and at the tip of filaments (Fig. 2H, b–g), and could clearly distinguish between undifferentiated thin and smooth filaments (Fig. 2H, a, e–g) and those that were broader with a heterogeneous and bulbous morphology that corresponded to differentiating filaments (Fig. 2H, a, g, h) (Chase *et al.*, 1976; Ferguson *et al.*, 1979). In addition, cell-wall remnants could be detected at the distal tip of differentiating filaments

where intracellular offspring had been released (Fig. 2H, h and Extended Data Fig. 1b) (Chase *et al.*, 1976). Together, these data demonstrate that *in vitro* culturing of SFB supports the full differentiation of *in vitro*-grown SFB filaments to the intracellular offspring stage and confirms the SFB life cycle inferred from *in vivo* observations.

Viability and infectivity of *in vitro*-formed intracellular offspring

SFB were first grown on TC7 cells under aerobic conditions (Fig. 3a) until many intracellular offspring appeared. After one additional day, intracellular offspring were no longer present and the average bacterial length was significantly longer, indicating intracellular offspring outgrowth into filaments. Similarly, when intracellular offspring grown *in vitro* were separated from filaments by filtration through a 5- μ m filter and added to newly plated cells, SFB numbers increased and the newly formed filaments differentiated into intracellular offspring at the filament tip (Fig. 3b, c), demonstrating filamentation and differentiation of *in vitro*-formed intracellular offspring. To assess whether SFB grown *in vitro* retained their ability to colonize mice and stimulate the characteristic innate and acquired immune responses, SFB grown *in vitro* for 3 days were divided into a filament/intracellular offspring fraction and a pure intracellular offspring fraction (Fig. 5a, b) and gavaged into germ-free mice. Colonization was firmly established by both inputs at 5 days after gavage (Fig. 5c) despite a 140-fold input difference for the filament/intracellular offspring and intracellular offspring fractions. However, unlike mice gavaged with SFB derived from faecal samples (SFB*Vivo*), which generally showed good colonization of the ileum, mice gavaged with *in vitro*-grown SFB (SFB*Vitro*) had much lower numbers of SFB colonizing the ileum (Fig. 3d and Extended Data Fig. 1d); instead they showed heavy colonization of the caecum (Fig. 5d). Thus, although SFB*Vitro* clearly can attach to the ileum, these results suggest that ileal colonization may be more efficient by intracellular offspring released from spores found in the faecal input, possibly because of the expression of flagella at this particular stage of the life cycle (Pamp *et al.*, 2012). Notably, the magnitude of the innate host response (Fig. 3e) was proportional to the colonization level of the ileum (Fig. 3e) and not the overall SFB faecal load (Extended Data Fig. 1c), revealing the requirement for ileal attachment of SFB to induce the innate host response. Similar to SFB*Vivo*, albeit with less potency, SFB*Vitro* were also able to stimulate the B-cell compartment in Peyer's patches (Fig. 3f, g), enhance IgA secretion in the faeces (Fig. 3h), and increase the number of Th17 cells and the level of IL-17A messenger RNA in the small intestine lamina propria (Fig. 3i-k).

SFB-host interaction and the host response to SFB growth.

Despite the apparent requirement for close contact between host cells and SFB for efficient growth, a tight interaction was not readily observed. However, when the interaction was promoted by gently spinning intracellular offspring onto cells, SFB filaments were found attached to mICcl2 cells (Fig. 4A-C). This stable interaction was accompanied by actin

accumulation surrounding the filament tip (Fig. 4C) and could leave structurally intact vacant attachment sites (Fig. 4A, c), similar to those observed in the ileum of mice (Fig. 5e). Attached filaments included both undifferentiated ones and those that had reached the final intracellular offspring stage. Contrary to *in vivo* results (Tannock *et al.*, 1984), attachment *in vitro* was not species specific (Fig. 4D). To assess the similarity in the host response to SFB *in vitro* and *in vivo*, it was analysed the gene expression profile of epithelial derived host factors known to be regulated by SFB colonization (Ivanov *et al.*, 2009; Gaboriau-Routhiau *et al.*, 2009; Goto *et al.*, 2014; Shima *et al.*, 2008; Lécuyer *et al.*, 2014). It was found that the gene regulation *in vitro* closely recapitulated gene regulation *in vivo* (Fig. 4E), thereby further supporting the *in vitro* model system. Going beyond previously implicated epithelial factors, it was tested several additional cytokines, chemokines, and host defence genes (Fig. 4F). The data show that SFB growth leads to a strong inflammatory host response, with the induction of pleiotropic inflammatory mediators such as tumour-necrosis factor- α (TNF- α), interleukin-1- α (IL1- α), and serum amyloid A 1-3 (Saa1-3), induction of several innate host defence mechanisms (Reg3 γ , iNos, and lactoferrin), and an immunological environment that is conducive for the recruitment of B cells, the transmigration of IgA, recruitment and activation of T cells, and recruitment of neutrophils, dendritic cells, as well as monocytes. In agreement, it was observed that the transcript level of most immune genes that are upregulated during co-culture with mICcl2 cells *in vitro* was also increased by SFB, and not by *Escherichia coli*, during colonization experiments *in vivo* (Fig. 5f). Conversely, the transcriptional response in the human TC7 cell line was divergent from that observed in mICcl2 cells and less consistent with *in vivo* results (Fig. 4g). Lastly, by using an array of microbe-associated molecular patterns (MAMPs), it was demonstrated that the inflammatory response to SFB *in vitro* is probably shaped by the activation of TLR2 (Fig. 4h).

Conclusion

These data demonstrate the successful culturing of SFB *in vitro* and provide new insights into SFB growth requirements and the host response to SFB challenge. These data suggest that *in vivo*, attachment of SFB to the ileal surface is an important feature to elicit epithelial cell responses, whereas *in vitro*, where attachment remained infrequent, the close proximity of SFB and cells appears largely to bypass the need for attachment to deliver the stimulating signal(s). These findings highlight the importance of the privileged location of the replicative niche of SFB at the ileal epithelial surface in mediating the stimulatory potential of SFB.

EXAMPLE 2: METHODS FOR GENETICALLY MODIFYING A SFB STRAIN

A series of modular shuttle vectors designed for use in Clostridia species (Heap *et al.*, 2009) are tested for their ability to be stably introduced into SFB during *in vitro* growth using both conjugation and transformation. Each plasmid contains a different origin of

replication with a choice of antibiotic resistance cassettes, multiple cloning sites and promoters. Conjugation and transformation are performed during *in vitro* growth of SFB.

Conjugation

An auxotrophic *E. coli* strain requiring the addition of Diaminopimelic acid to the medium for growth is used as a donor strain as this strain can be easily selected against in rich medium with the absence of diaminopimelic acid.

SFB strain is purified from monoassociated mice as described above and intracellular offsprings (IOs) are added to host cells (TC7 cells) grown on either tissue culture or transwell plates under conditions described for SFB growth above.

After substantial growth has occurred (2-4 days), diaminopimelic acid (100 ug/ml) and the donor *E. coli* strain (requiring dap addition for growth and carrying a plasmid) is added and centrifuged together with cells and SFB and maintained for a given time (*e.g.*, 4-8 hours).

Alternatively, *in vitro* grown (cultured) SFB are collected and mixed with the aforementioned *E.coli* strain and pelleted (all under low oxygen) and maintained in a pellet for varying amounts of time (*e.g.*, 4-8 hours).

Bacteria are collected and washed 2x with regular SFB growth medium lacking diaminopimelic acid before being put on fresh eukaryotic cells (always at low oxygen) with regular SFB medium but complemented with the appropriate antibiotic to which the conjugal plasmid gives SFB resistance to.

After a given time (0-24 hours), SFV are collected and gavaged into germfree mice.

Recombinant SFB are selected for and maintained by antibiotic treatment of the drinking water. The optimal and minimal regime of antibiotic use in the drinking water that is needed to cure SFB monoassociated mice from SFB is determined prior and used to select for recombinant SFB.

Transformation by electroporation

As described above, SFB are grown (cultured) *in vitro* for 2-4 days on TC7 cells.

SFB are collected and put on ice. Under low oxygen conditions and working with solutions put on ice, SFB are washed 6x with 10% glycerol/pre-equilibrated water before being resuspended and concentrated with 10% glycerol/H₂O.

Purified plasmid DNA is added and SFB is electroporated (6.5 kV.cm⁻¹ to 25kV.cm⁻¹).

SFB are placed in regular SFB medium again and spun onto fresh cells. SFB are allowed to recover for 3-24 hours before selective antibiotic be added overnight.

SFB are gavaged into GF mice.

Recombinant SFB are selected for and maintained by antibiotic treatment of the drinking water.

Transformation by the chemical method: calcium chloride and heat shock

As described above, SFB are grown *in vitro* for 2-4 days on TC7 cells.

SFB are collected and put on ice. Under low oxygen conditions and working with solutions put on ice, SFB are washed 5x with 0.1M calcium chloride before being resuspended and concentrated with 10% glycerol/0.1M calcium chloride.

Purified plasmid DNA is added to SFB and incubated for 30 minutes.

SFB are heat shocked at 42°C for 30 seconds before being placed in regular SFB medium again and spun onto fresh cells. SFB are allowed to recover for 3-24 hours before selective antibiotic be added overnight.

SFB are gavaged into GF mice.

Alternatively, the above procedure may be performed at room temperature only without heat shock.

Recombinant SFB are selected for and maintained by antibiotic treatment of the drinking water.

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CLAIMS

1. An *in vitro* method of culturing a segmented filamentous bacterium strain, comprising co-culturing said segmented filamentous bacterium strain with a eukaryotic host cell, wherein the culture is performed at an O₂ level inferior to 5% in a rich tissue culture liquid medium containing bacterial medium components including iron.
2. An *in vitro* method of culturing a segmented filamentous bacterium strain, comprising the steps of:
 - a) growing a eukaryotic host cell on a solid culture medium;
 - b) immersing the eukaryotic host cells grown in step a) in a eukaryotic host cell-SFB liquid culture medium;
 - c) challenging the cell culture of step b) with a SFB strain, preferably an intracellular SFB offspring;
 - d) co-culturing the live eukaryotic host cell and the SFB strain in the liquid culture medium at an O₂ level inferior to 5%; and optionally
 - e) recovering the cultured SFB strain.
3. The method according to claim 1 or claim 2, wherein the eukaryotic host cell is an epithelial cell or a cancer cell.
4. The method according to any one of claim 1 to 3, wherein the eukaryotic host cell is a cell from the gastrointestinal tract or a carcinoma cell.
5. The method according to any one of claim 1 to 4, wherein the eukaryotic host cell is selected from the group consisting the human Caco-2, TC7 or HeLa cell lines or mouse mICcl2 or CMT93 cell lines.
6. The method according to any one of claim 2 to 5, wherein in step a) the eukaryotic host cell is grown at an O₂ level comprised between 0% and 5%, preferably between 0.5 and 3%, more preferably between 1 and 2.5%.
7. The method according to any one of claims 2 to 6, wherein in step b) the eukaryotic host cell-SFB liquid culture medium is a rich tissue culture medium containing bacterial medium components including iron.
8. The method according to any one of claims 1 to 7, wherein the liquid culture medium comprises DMEM/F12 advanced medium, supplemented with fetal calf serum, non-essential amino acids, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, brain-heart infusion, a yeast/peptone/casein amino-acid mixture and iron.
9. The method according to any one of claim 1 to 8, wherein in step b) the SFB strain is in the form of a filament or an intracellular offspring.
10. The method according to any one of claim 2 to 9, wherein in step c), the SFB strain is closely contacted with or in directly contacted with the eukaryotic host cell.

11. The method according to any one of claim 1 to 10, wherein the co-culture of the eukaryotic host cell and the SFB is performed at an O₂ level comprised between 0% and 5%, preferably between 0.5 and 3%, more preferably between 1 and 2.5%.

12. The method according to any one of claim 2 to 11, wherein the cultured SFB strain is recovered in a form of a filament, an intracellular offspring or a spore.

13. A method for genetically modifying a segmented filamentous bacterium strain by conjugation, comprising the steps of:

i) genetically modifying an *Escherichia coli* strain with a recombinant shuttle vector DNA comprising a DNA sequence of interest to introduce in the SFB strain, wherein the said shuttle vector DNA is capable of replicating in the *E. coli* strain, and in the SFB strain to be targeted for genetic modification;

ii) co-culturing the genetically modified *E. coli* strain obtained in step i) together with the SFB strain to be targeted for genetic modification according to *in vitro* method of culturing of any one of claims 2 to 12, wherein:

in step c) of said method of culturing, the cell culture of step b) is challenged with both the SFB strain to be targeted for genetic modification, preferably an intracellular SFB offspring, and said genetically modified *E. coli* strain; and

in step d) of said method of culturing, the live eukaryotic host cell, the SFB strain and the genetically modified *E. coli* strain are co-cultured in the liquid culture medium at an O₂ level inferior to 5%;

iii) recovering the genetically modified SFB strain of step ii).

14. A method for genetically modifying a segmented filamentous bacterium strain by electroporation, comprising the steps of:

i) culturing and recovering a segmented filamentous bacterium strain according to the *in vitro* method of culturing of any one of claims 2 to 12;

ii) mixing the SFB strain recovered in step i) with a purified plasmid to be transformed, preferably in glycerol/H₂O;

iii) applying an electric pulse to the mixture obtained in step ii);

iv) recovering the genetically transformed SFB strain.

15. A method for genetically modifying a segmented filamentous bacterium strain by chemical transformation, comprising the steps of:

i) culturing and recovering a segmented filamentous bacterium strain according to the *in vitro* method of culturing of any one of claims 2 to 12;

ii) mixing the SFB strain recovered in step i) with a purified plasmid to be transformed, preferably in glycerol/calcium chloride;

iii) incubating the mixture obtained in step ii);

iv) optionally applying a heat shock to the mixture;

v) recovering the genetically transformed SFB strain.

16. An isolated genetically modified SFB strain expressing an antigen of interest.

17. A live genetically modified SFB strain expressing an antigen of interest for use as a medicament, preferably for use in preventing or treating a disease caused by a microorganism from which said antigen derives or a cancer.

5 18. A therapeutic immunogenic composition comprising a live genetically modified SFB strain expressing an antigen of interest.

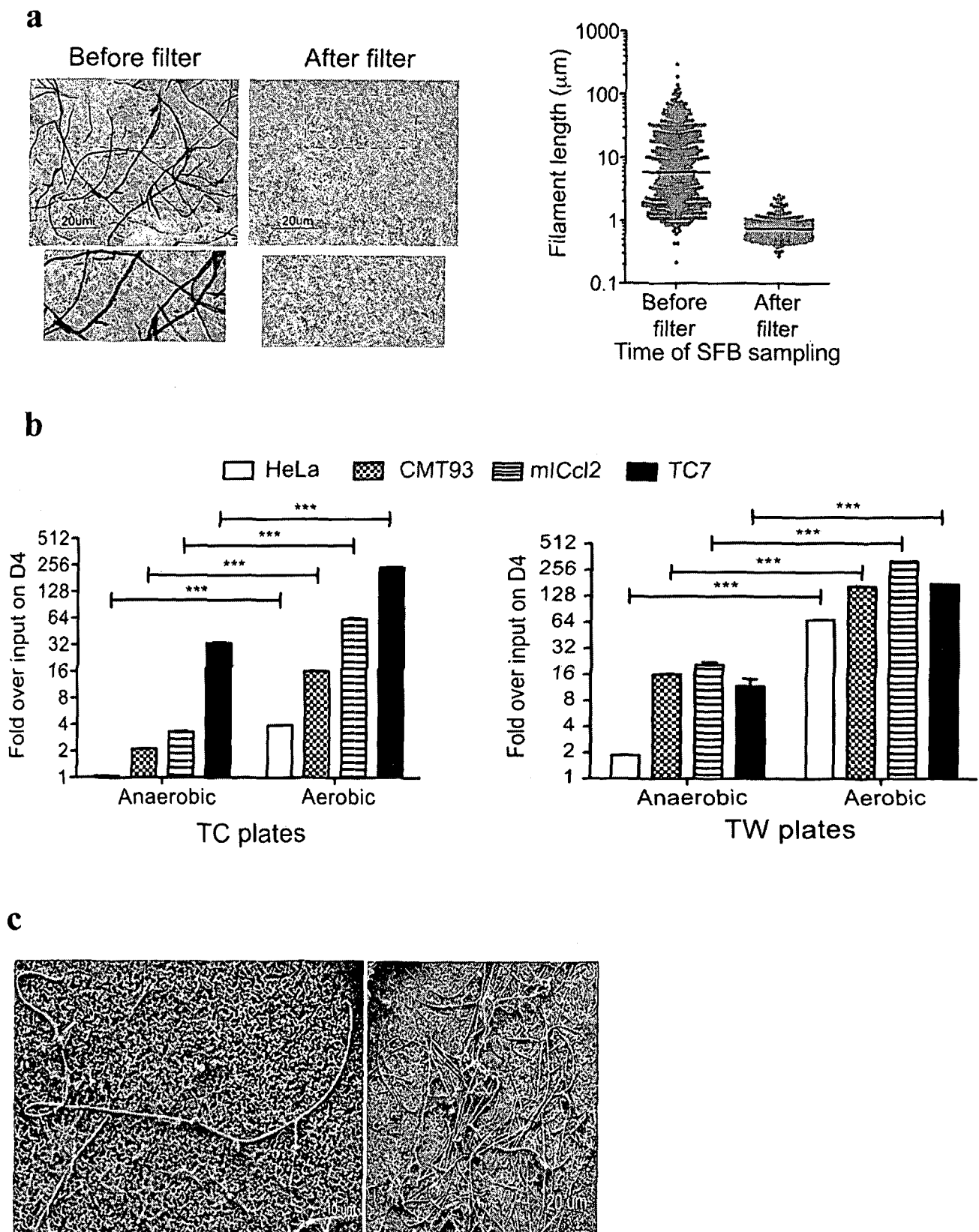


Figure 1

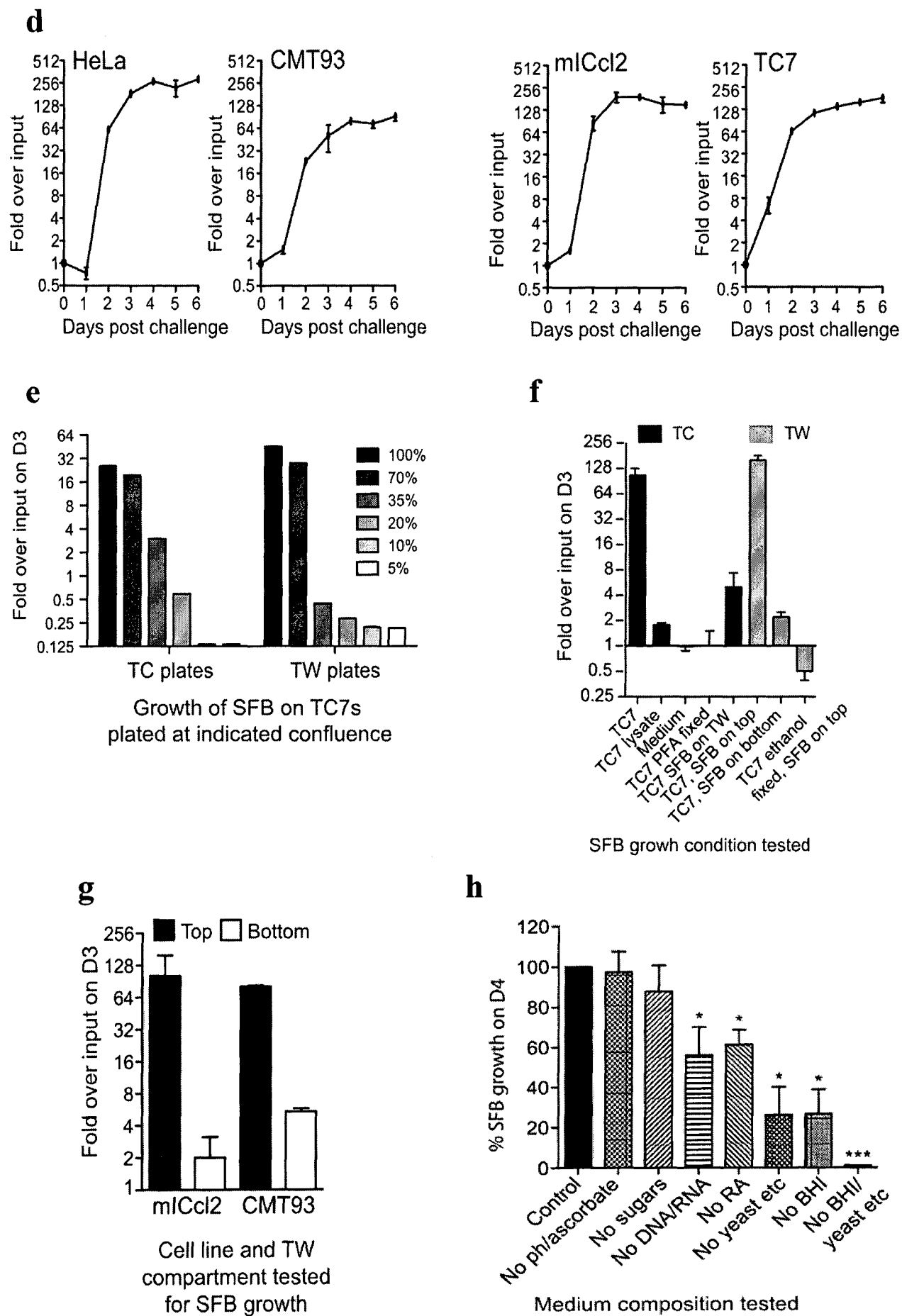
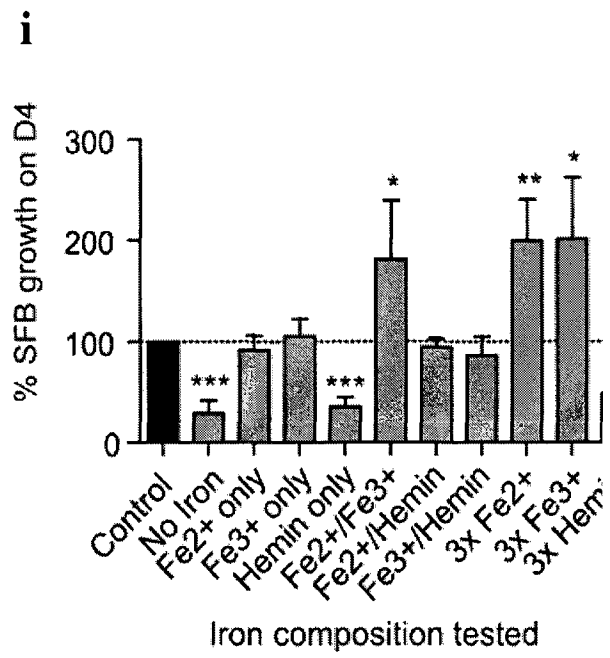


Figure 1

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**Figure 1**

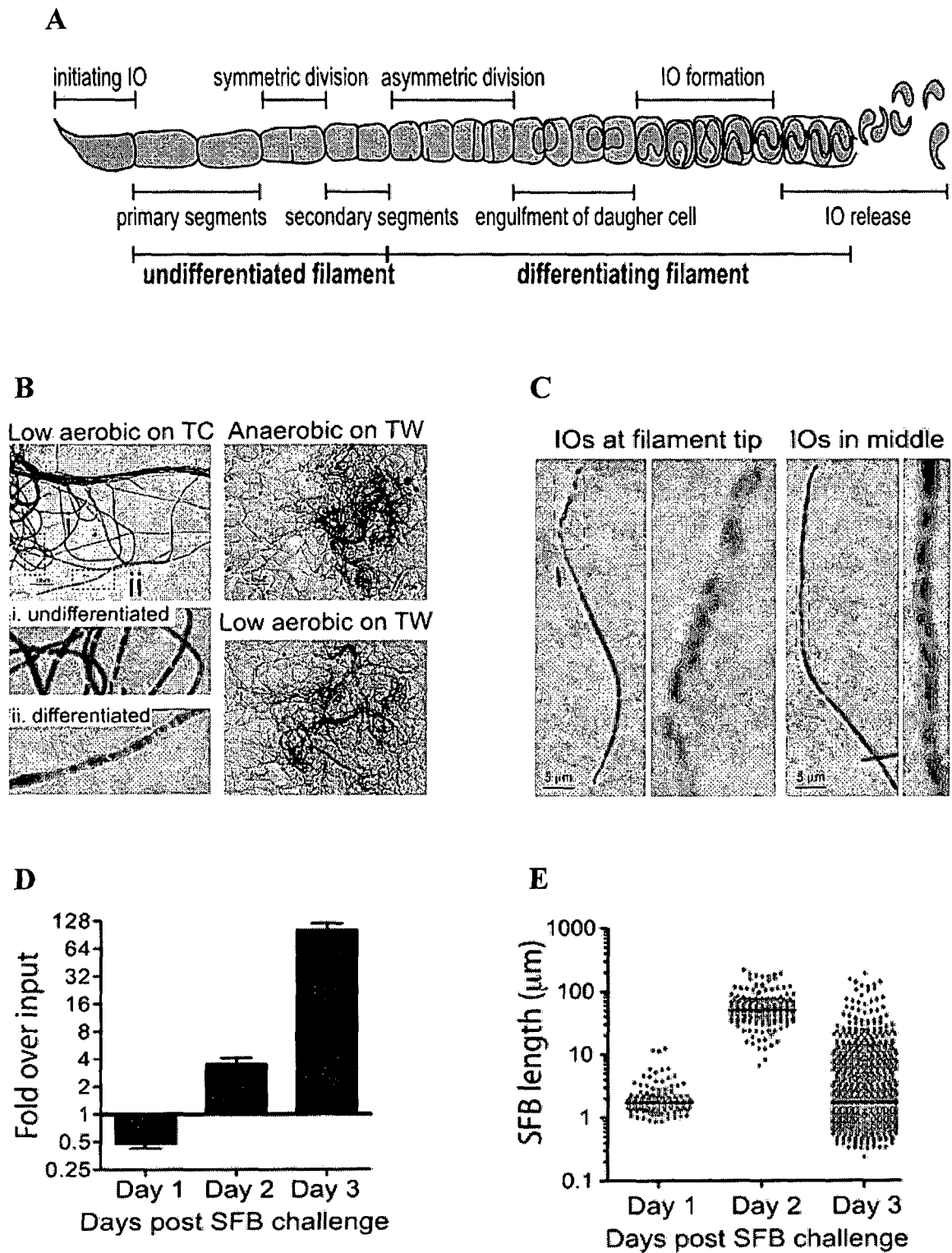
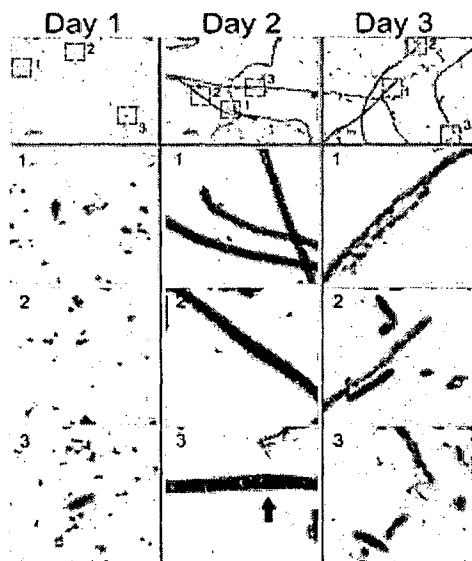
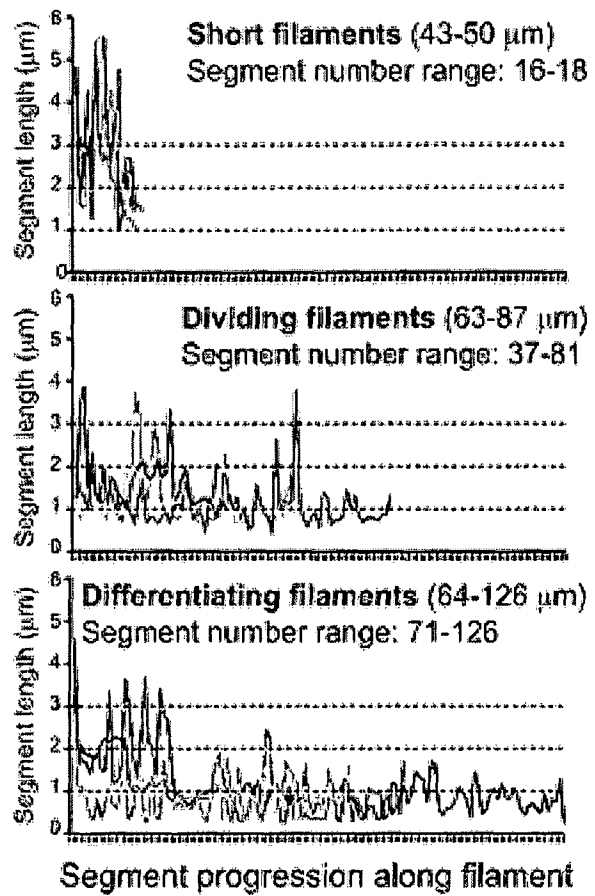
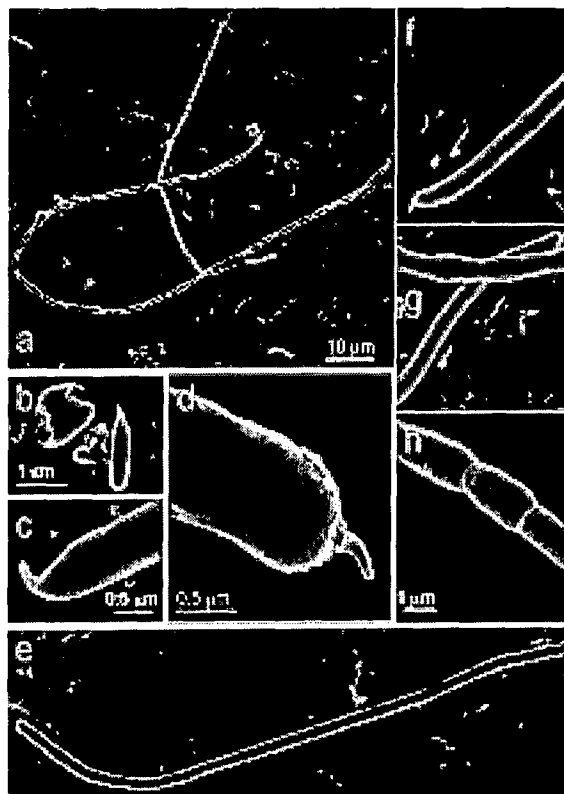


Figure 2

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F**G****H****Figure 2**

SUBSTITUTE SHEET (RULE 26)

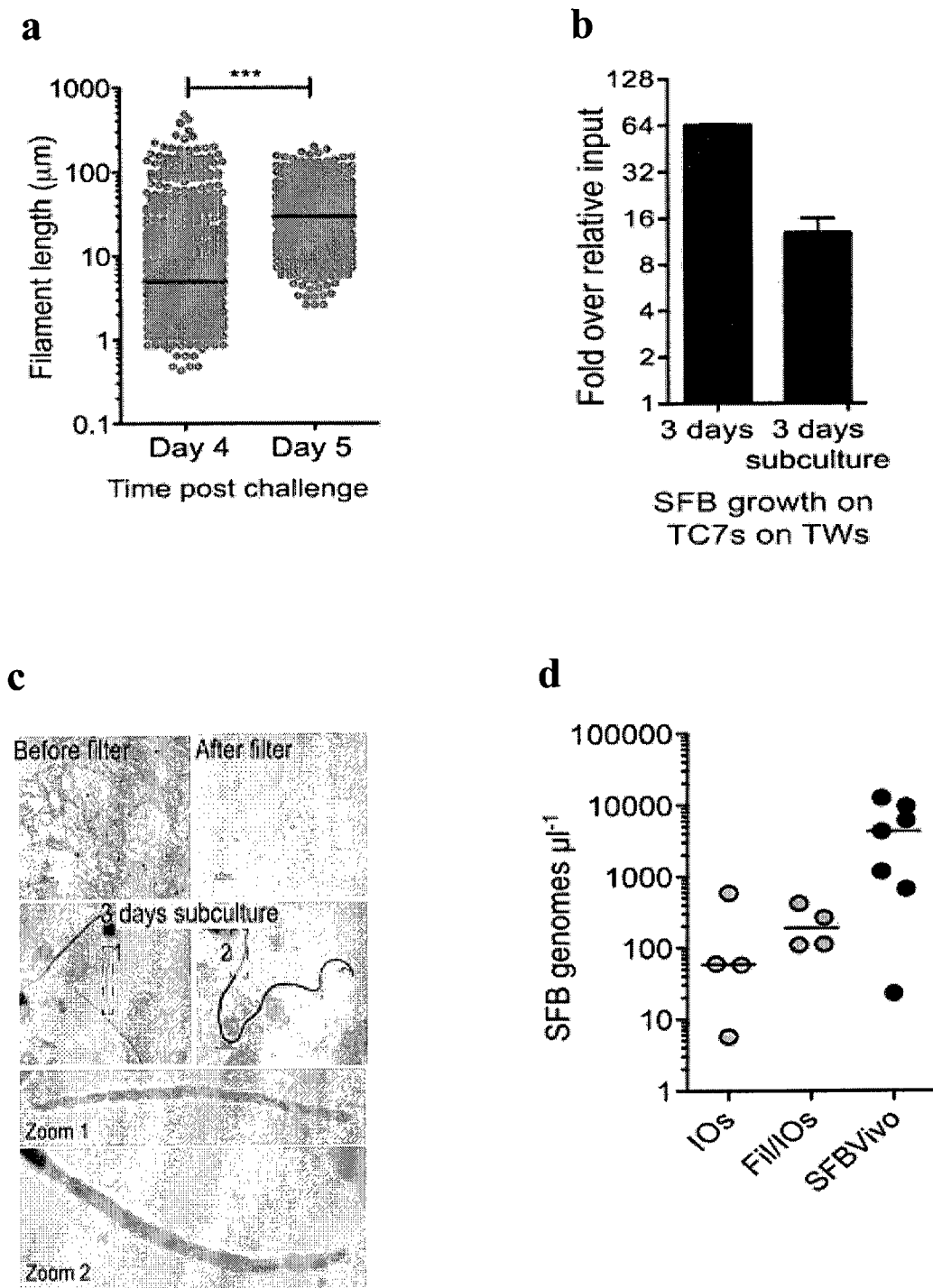
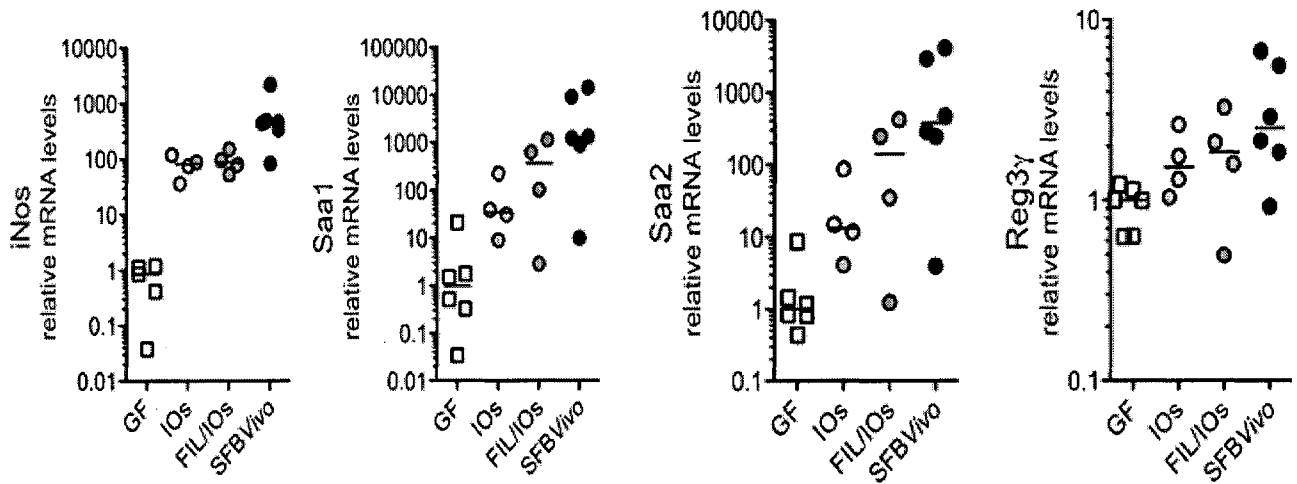
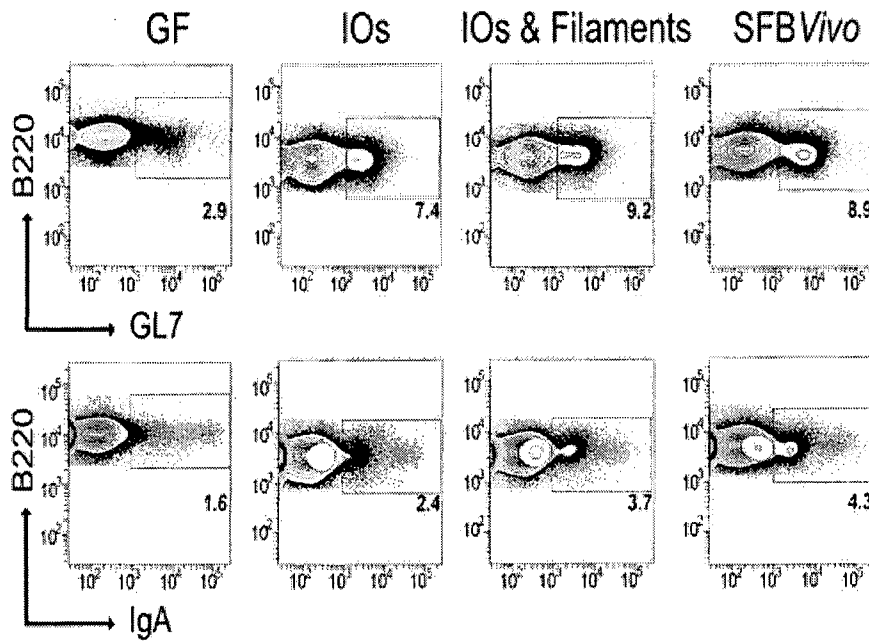


Figure 3

e



f



g

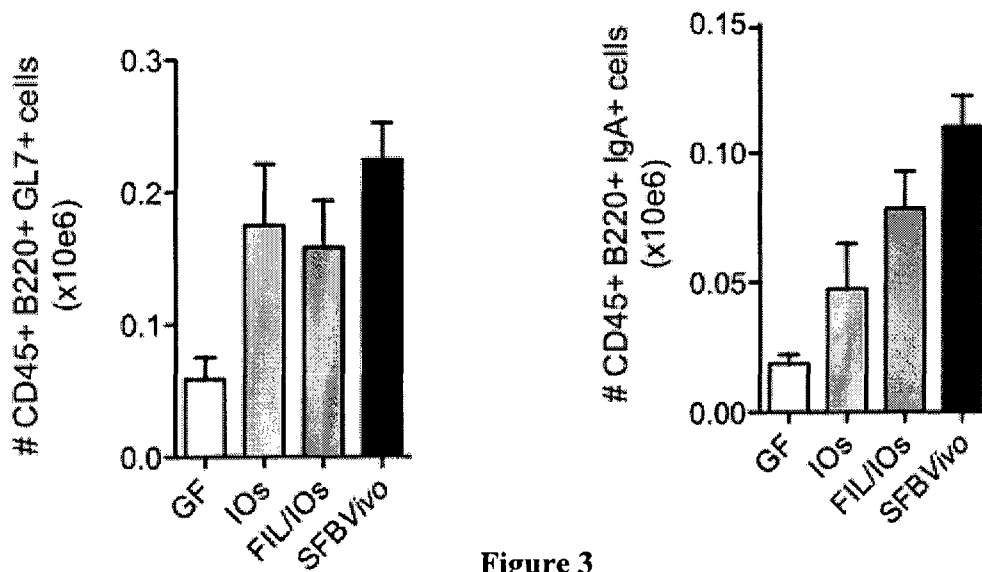


Figure 3

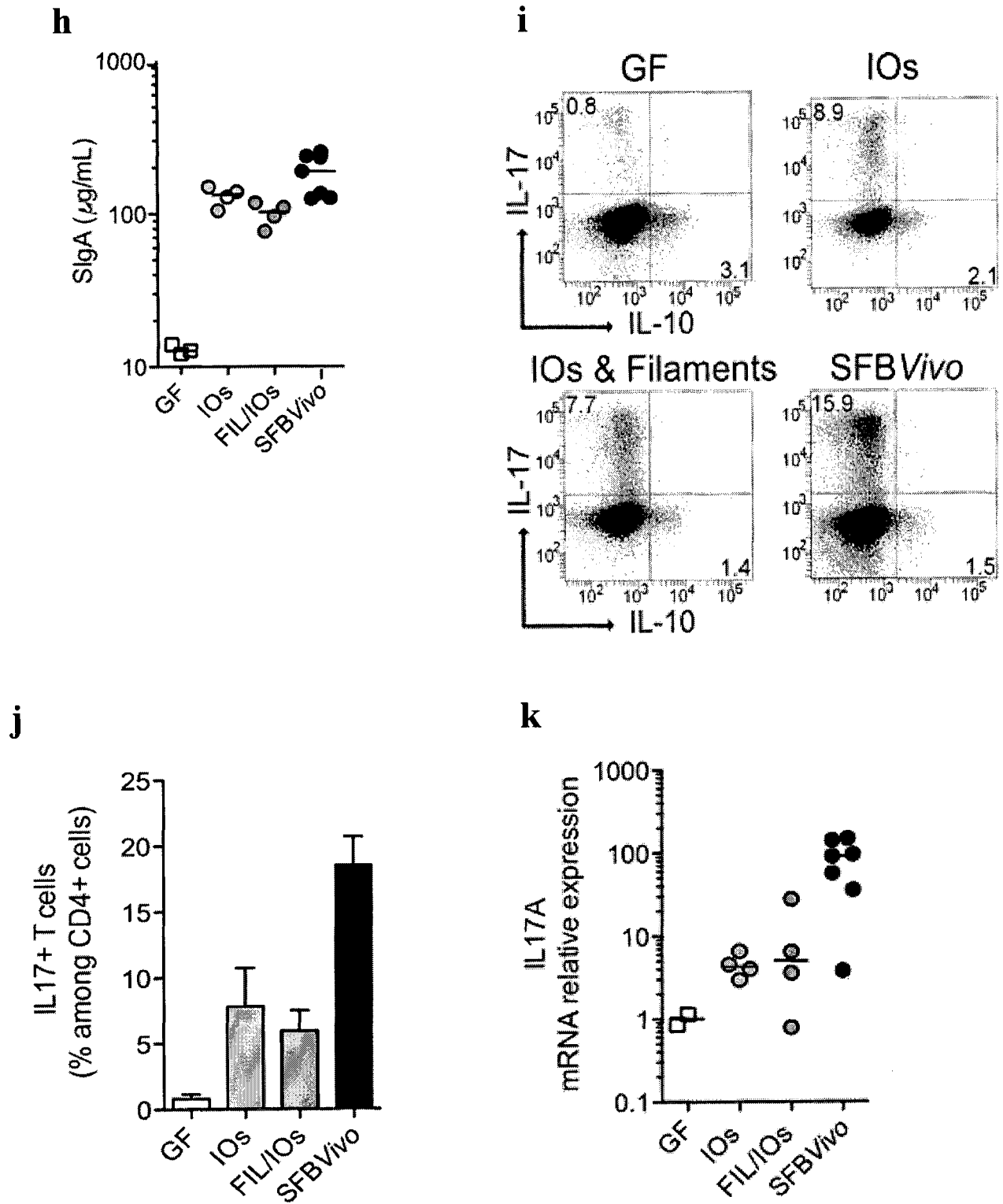
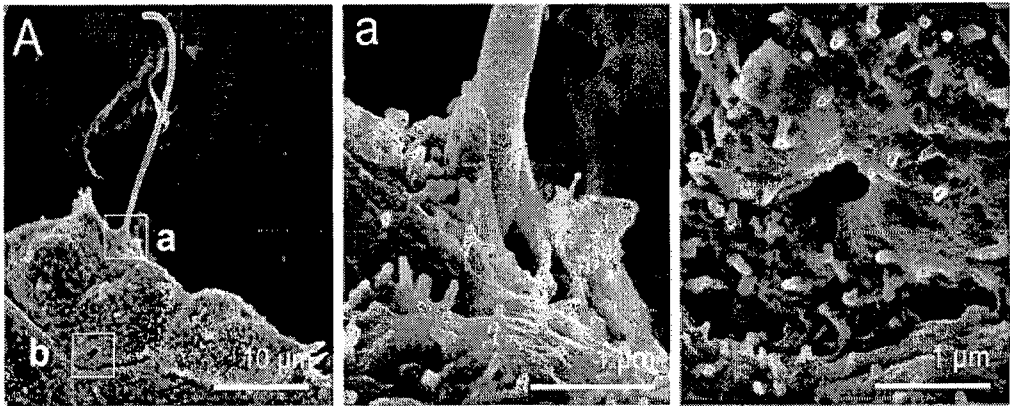
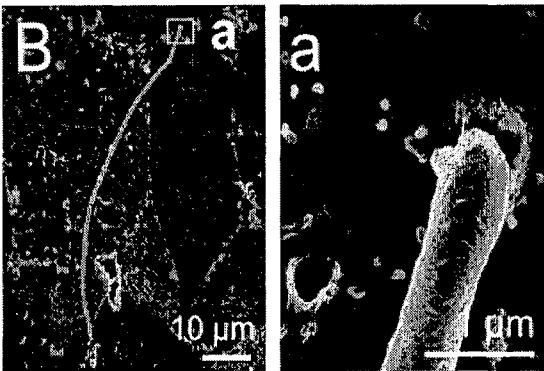


Figure 3

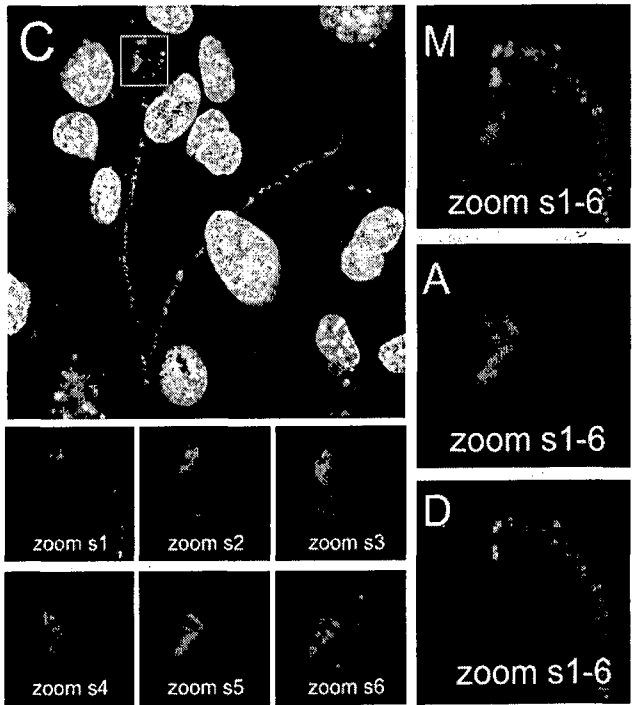
A



B



C



D



E

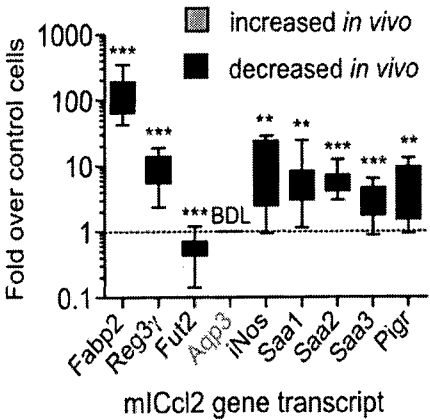
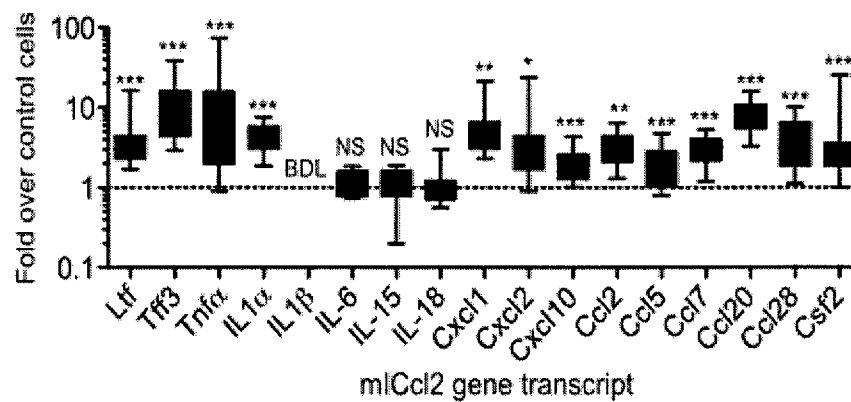


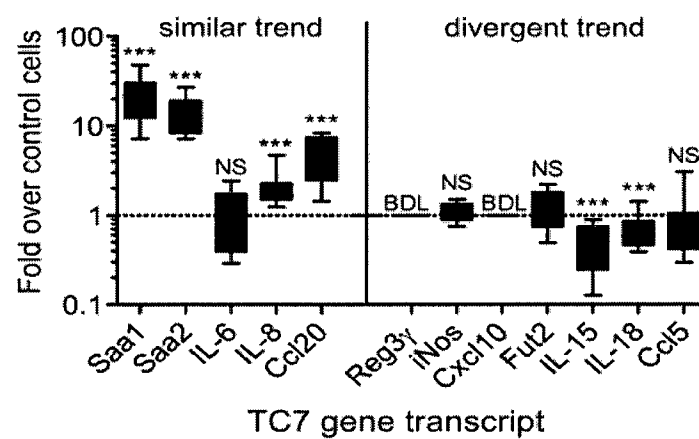
Figure 4

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F



G



H

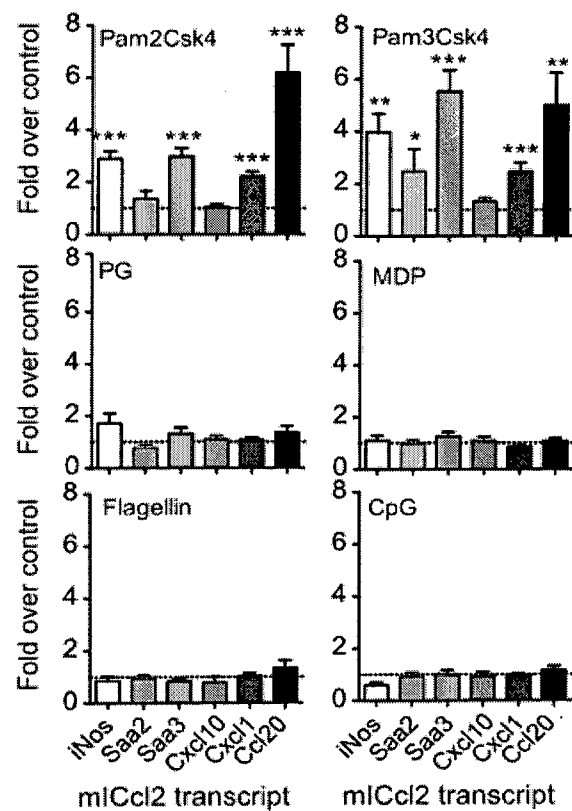


Figure 4

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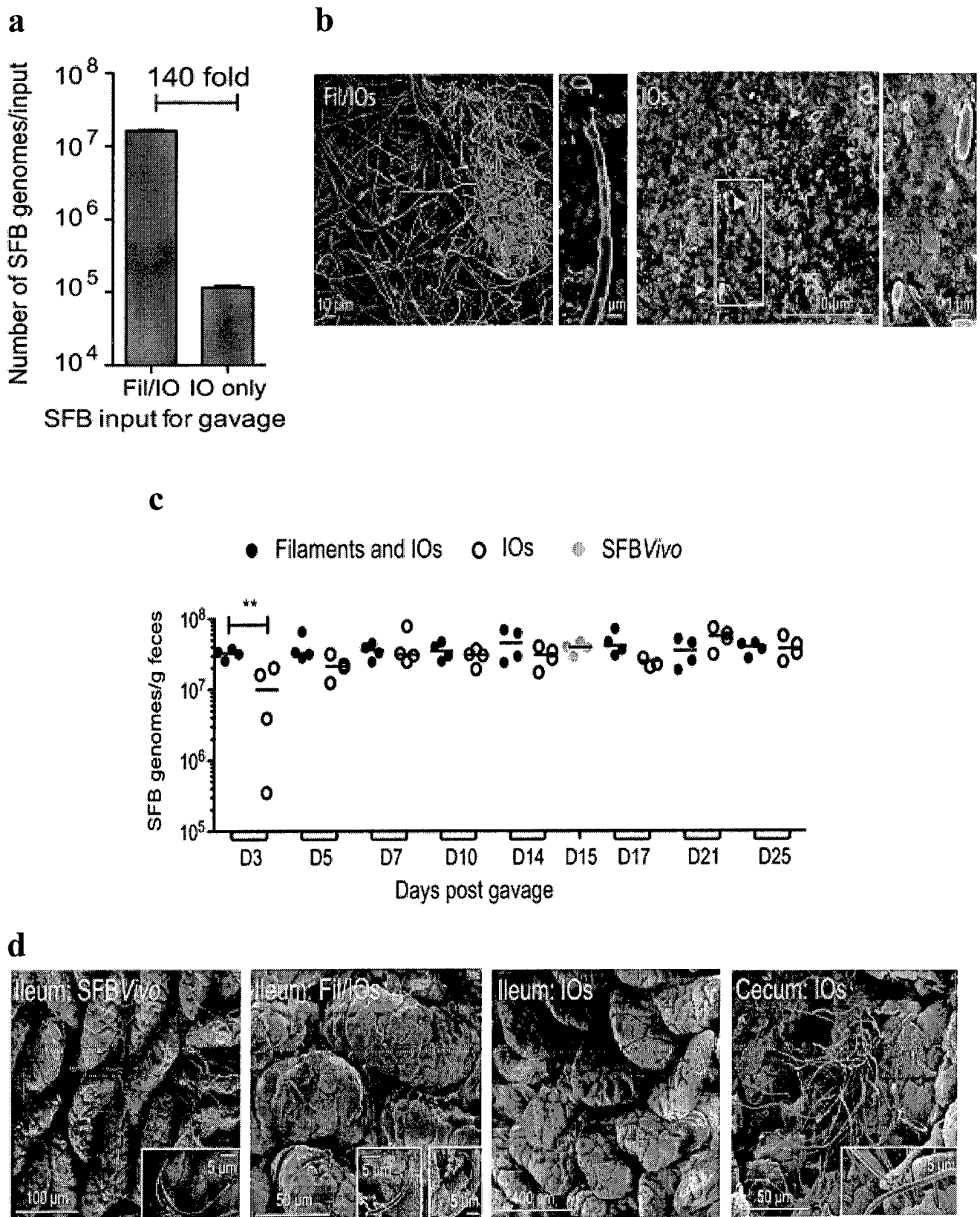
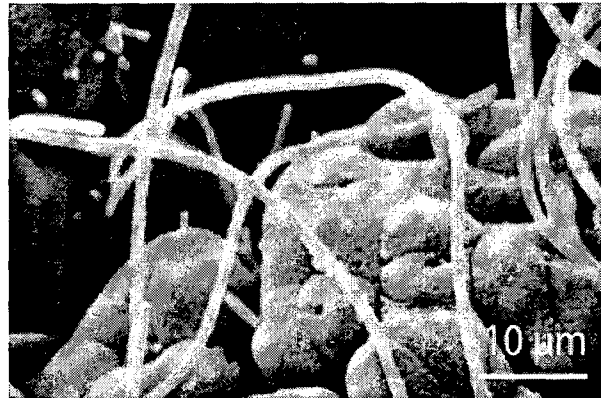
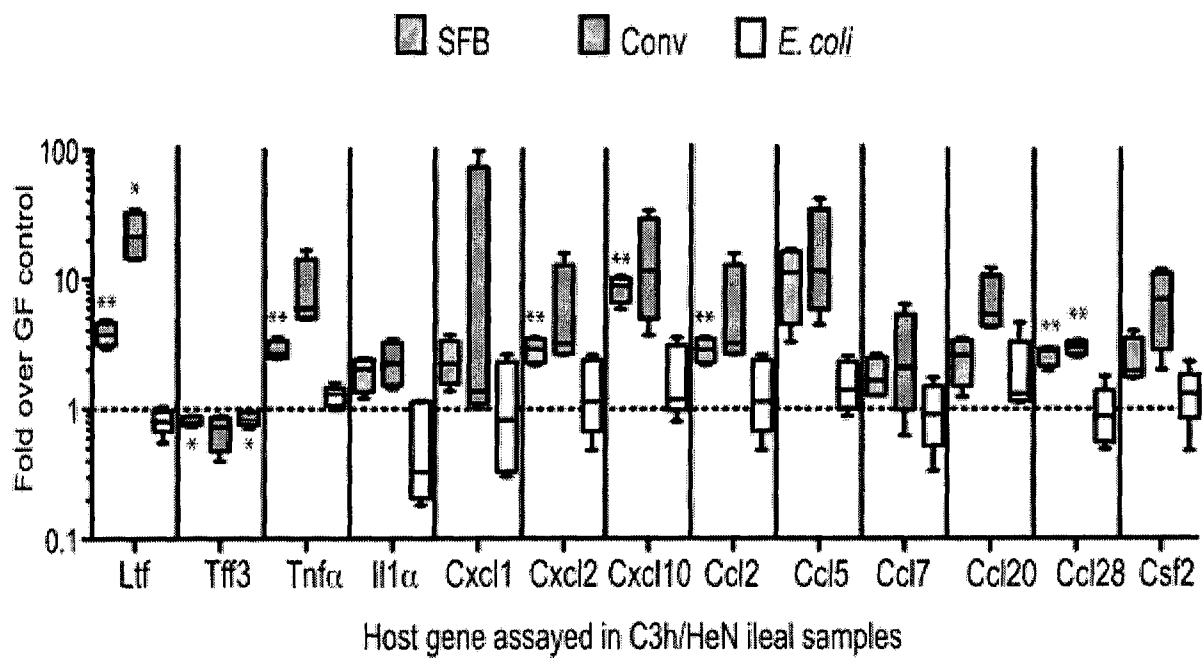


Figure 5

e**f****Figure 5**

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2014/067285

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N1/20
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/046616 A2 (UNIV NEW YORK [US]; UNIV CALIFORNIA [US]; LITTMAN DAN [US]; IVANOV IV) 21 April 2011 (2011-04-21)	16-18
Y	the whole document claim 5	1-15
Y	----- E. J. STEWART: "Growing Unculturable Bacteria", JOURNAL OF BACTERIOLOGY, vol. 194, no. 16, 1 June 2012 (2012-06-01) , pages 4151-4160, XP55198147, ISSN: 0021-9193, DOI: 10.1128/JB.00345-12 the whole document page 4156, column 2 - page 4158, column 2 ----- -/--	1-15

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

☒ See patent family annex.

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"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

26 June 2015

Date of mailing of the international search report

06/07/2015

Name and mailing address of the ISA/

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Sprinks, Matthew

INTERNATIONAL SEARCH REPORT

International application No

PCT/IB2014/067285

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	S. J. PAMP ET AL: "Single-cell sequencing provides clues about the host interactions of segmented filamentous bacteria (SFB)", GENOME RESEARCH, vol. 22, no. 6, 20 March 2012 (2012-03-20), pages 1107-1119, XP55198149, ISSN: 1088-9051, DOI: 10.1101/gr.131482.111 the whole document page 1116, column 1 -----	1-15
Y	MITSUTOSHI SENOH ET AL: "Conversion of viable but nonculturable enteric bacteria? to culturable by co-culture with eukaryotic cells", MICROBIOLOGY AND IMMUNOLOGY, vol. 56, no. 5, 27 April 2012 (2012-04-27), pages 342-345, XP55198160, ISSN: 0385-5600, DOI: 10.1111/j.1348-0421.2012.00440.x the whole document page 345, column 1 -----	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/IB2014/067285

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011046616 A2	21-04-2011	US 2012276149 A1 WO 2011046616 A2	01-11-2012 21-04-2011
