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# Mono-association of mice with non-cultivable, intestinal, segmented, filamentous bacteria

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**Abstract.** A technique is described so that mice mono-associated with non-cultivable, segmented filamentous bacteria (SFB's) can be produced for the first time. As SFB donors, mice were used which had an intestinal microflora consisting of both SFB's and bacteria of the genus *Clostridium*. Recipients were germ-free mice. It was demonstrated that the intraileal inoculation method was more effective than the orogastric route. Therefore, intestinal homogenates of donor mice were treated with filtered ethanol, diluted and administered intraileally to recipient mice. Evidence is presented that cage mates of the recipient mice were mono-associated with SFB's. The availability of these animals, i.e. in vivo monocultures of SFB's, allows taxonomic and functional characterization of SFB's, which was as yet not possible.

**Key words:** Germ-free mice – Intestinal bacteria – Mouse – Segmented – Filamentous bacteria

Autochthonous, segmented, filamentous bacteria (SFB's) inhabiting the ileum of mice and rats have been extensively studied morphologically by scanning and transmission electron microscopy (Davis and Savage 1974; Savage and Blumershine 1974; Chase and Erlandsen 1976; Blumershine and Savage 1978; Snellen and Savage 1978; Ferguson and Birch-Andersen 1979; Koopman et al. 1987). Functional characterization and identification of the bacterium have been hampered because attempts to culture SFB's in vitro were unsuccessful (Savage and Blumershine 1974; Koopman et al. 1984, 1988; Merrell et al. 1979; Tannock et al. 1987). In addition, the possible significance of SFB's to the host remains unclear. There are some indications that SFB's may play a role in the gastrointestinal colonization resistance against pathogenic invaders (Merrell et al. 1979; Garland et al. 1982; Koopman et al. 1984).

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*Abbreviation:* SFB, Segmented filamentous bacterium

In a preliminary attempt to produce mice mono-associated with SFB's we prepared intestinal homogenates derived from specified pathogen-free (SPF) mice colonized with SFB's, treated these homogenates with ethanol and chloroform and administered dilutions of treated homogenates to germ-free mice (Klaasen et al. 1990b). This procedure resulted in mice associated with both SFB's and clostridia. With the use of these mice as SFB donors, we now describe a technique to produce mice mono-associated with SFB's.

## Materials and methods

*Animals and housing.* Germ-free, Cpb:SE (Swiss) mice of both sexes, aged 4 weeks, and kept in a Trexler type plastic isolator under conditions as described (Klaasen et al. 1990b), were used throughout as SFB recipients. Donor mice used to determine the minimum dose of SFB's necessary to colonize the ileum of recipient mice (experiment 1) were female Cpb:SE mice, aged 5 weeks, with an SPF flora. Microbiological quality of these mice and their housing conditions have been described elsewhere (Koopman et al. 1989b). SFB donor mice used to convert germ-free mice into mice mono-associated with SFB's (experiment 2) were 5 week-old Cpb:SE mice of both sexes with an intestinal microflora consisting of SFB's and some bacterial species from the genus *Clostridium*. These donor mice were produced and housed as described (Klaasen et al. 1990b).

After inoculation of the recipient mice in experiment 1, they were housed individually on sterilized commercial sawdust (Woody-Clean type $^3/_4$, J. Rettenmaier und Söhne, Ellwangen-Holzmühle, FRG) in type S macrolon cages (RUCO Metaalindustrie Nederland BV, Valkenswaard, The Netherlands), provided with a filter cap. In experiment 2, the inoculated mice were placed back into the isolator and housed together with untreated, germ-free counterparts in type III macrolon cages. Environmental conditions in the isolator were as described earlier (Klaasen et al. 1990b).

All mice received a sterilized, pelleted diet (Koopman et al. 1989a) and sterilized, demineralized water *ad libitum*.

### *Experiment 1. Determination of minimum "infectious" dose of SFB's and effective route of inoculation*

*Preparation of intestinal homogenates.* Eight SPF donor mice were killed by cervical dislocation. From each mouse, part of the small

**Table 1.** Colonization of the small intestine of germ-free mice by administered segmented, filamentous bacteria (SFB's)

| Number of SFB's administered[a] | Route of administration | SFB score[b] in recipients at 1 week after inoculation | Incidence[c] of SFB-positive recipients |
|---|---|---|---|
| $2.0 \times 10^2$ | orogastric | $0 \pm 0$ | 0/4 |
| $2.0 \times 10^4$ | idem | $29 \pm 10$ | 4/4 |
| $4.0 \times 10^1$ | intraileal | $0 \pm 0$ | 0/4 |
| $2.0 \times 10^2$ | idem | $38 \pm 9$ | 4/4 |
| $2.0 \times 10^4$ | idem | $39 \pm 22$ | 4/4 |

[a] Number of SFB's given per mouse as based on microscopic counts in the diluted intestinal homogenates.
[b] Percentage of SFB-positive fields as determined in mucosal smears of the distal small intestine; means ± SD for 4 mice per treatment.
[c] Number of SFB-positive mice/number of examined mice

intestine, with a length of 12 cm and the lower end being located at 1 cm from the cecum, was removed. The intestinal contents were gently removed with a pair of tweezers and the pooled intestines (0.7 g, wet weight) homogenized in 4.5 ml of broth (Koopman et al. 1983) with the use of an Ultraturrax homogenizer (TP 18.10; Janke and Kunkel, Staufen im Breisgau, FRG). This homogenate was stepwise diluted by a factor 10 up to 6 times using the broth as diluent. The concentration of SFB's in the dilutions was determined as follows. 0.01 ml of dilution was spread on 1 $cm^2$ of a microscopic slide and Gram-stained. With a light microscope, the number of SFB's was counted in 20 fields at 4 randomly chosen spots of the slide at a magnification of 1000×. With the mean number of SFB's per field, the number of SFB's per ml of each dilution was calculated.

*Inoculation.* SFB's were administered to germ-free mice either orogastrically or intraileally. Orogastric inoculation was performed with a blunt needle. Two different SFB doses (Table 1) were given, the inoculum volume being 0.5 ml/mouse. Intraileal inoculation was carried out as described below in experiment 2. 0.1 ml of broth containing one of three different doses (Table 1) was injected into the lumen of the ileum, at 0.5 cm from the cecum.

*Examination of inoculated mice.* Seven days after inoculation the recipient mice were killed by cervical dislocation. The small intestinal mucosa was examined for the level of colonization of mucosa-associated SFB's. Mucosal smears on microscopic slides were prepared as described by Klaasen et al. (1990a). With a light microscope, the colonization density of SFB's was determined in smears derived from five different locations (Klaasen et al. 1990a) in the distal half of the small bowel, and expressed as SFB score. This score is the mean percentage of SFB-positive fields out of 20 fields per smear examined at a magnification of 1000×.

## *Experiment 2. Mono-association of germ-free mice with SFB's*

*Preparation and treatment of intestinal homogenates.* Ten donor mice with a microflora of SFB's and clostridia were killed by cervical dislocation while in the isolator. They were then transferred, strictly aseptically, into a sterile laminar flow cabinet to collect the small intestines. From pooled small intestine sections (0.8 g, wet weight) one homogenate was prepared by adding 4.5 ml of broth as described above under experiment 1, except that it was done under strictly aseptic conditions. From the mucosal regions located proximally and distally adjacent to the intestinal section removed, mucosal smears were prepared and the SFB scores determined as described (Klaasen et al. 1990a). The concentration of SFB's in the homogenate was determined as described above under experiment 1. The homogenate was treated as follows. Ethanol (96%), which had been passed through a millipore filter (FP030/3, Schleicher and Schuell, Dassel, FRG) to eliminate any spore-forming bacteria, was added to the homogenate (ethanol : homogenate = 19:1, v/v) to a final concentration of 91%. This mixture was incubated for 4 h at 20° C while stirring with a Morat magnetic stirrer (Franz Morat KG, Framo-Gerätetechnik, Hochschwarzwald, FRG). Then, the ethanol was separated by centrifugation at 600× g during 20 min. The ethanol supernatant was carefully removed and the pellet resuspended in 4.5 ml of broth (Koopman et al. 1983). Two portions (0.5 ml each) of this suspension were diluted with the broth in tenfold dilution steps up to 7 times. The concentration of SFB's in the undiluted suspension was determined as described above. Except for centrifugation, all procedures were carried out in a sterile laminar flow cabinet.

*Selection of proper dilution for inoculation.* Samples (0.1 ml) of the ethanol-treated, undiluted homogenate and of one complete dilution series were added in duplicate onto pre-reduced, enriched blood agar plates (Koopman et al. 1973). The plates as well as the homogenate and the dilutions were then incubated for 18 h at 37° C in an anaerobic glove box (ITL International BV, Leek, The Netherlands) as described by Koopman et al. (1973). Samples (0.1 ml) of the other dilution series were added in duplicate onto non pre-reduced, but otherwise identical, agar plates. These dilutions and plates were incubated aerobically for 18 h at 37° C. After incubation all samples were examined for bacterial growth, followed by repetition of the subculturing procedure. After another 24 h all samples were examined again. To check the influence of incubation on SFB concentration, this concentration was determined in the ethanol-treated, undiluted homogenate before and after anaerobic incubation at 37° C for 42 h. The lowest dilution but one, without visible bacterial growth after either anaerobic or aerobic incubation and with sufficiently high concentration of SFB's for an effective intraileal administration was selected for inoculation of germ-free mice. This dilution was taken from the anaerobically incubated series. After concentrating this dilution which was the $10^4$-fold diluted homogenate, by centrifugation and resuspending the pellet in 1.0 ml of broth, the concentration of SFB's was determined as described above.

*Intraileal inoculation.* Two germ-free mice were transferred under aseptic conditions from an isolator into a sterile laminar flow cabinet. They were anesthesized as follows. 0.1 ml of 10-fold with saline diluted Hypnorm (Janssen Pharmaceutica, Tilburg, The Netherlands), containing 10 µg of fluanisone and 0.315 µg of phentanyl citrate, and 0.1 ml of 10-fold with saline diluted Valium 10 Roche (Hoffmann-La Roche BV, Mijdrecht, The Netherlands), containing 50 µg of diazepam, were separately injected intramuscularly. After general anesthesia had been achieved, the abdomen was opened and at 0.5 cm from the ileocecal junction an inoculum of 0.1 ml was injected into the ileal lumen. Then, the abdomen was closed by a suture of the muscle layer with silk (USP 3-0); the skin was closed with two suture clips. The two mice were transferred back, strictly aseptically, to the isolator. They were allowed to recover in abdominal position while hypothermia was prevented by placing a heating element (38° C) directly under the bottom of the isolator. After 5 h of recovery, each mouse was placed in a cage containing 6 germ-free mice. The germ-free status of these animals had been investigated previously by bacteriological and microscopic examination of pooled samples of feces from the isolator. In addition, one of their cage mates had been killed to examine its intestine for the presence of (in vitro) cultivable or non-cultivable bacteria. Both examinations resulted in the absence of bacteria which implied that SFB's were not present in the isolator previously to the experiment.

*Examination of mice exposed to inoculated counterparts.* Four mice of which two had been housed with one inoculated mouse and two with the other, were examined either 5, 10 or 15 days after inoculation. For this purpose, they were killed by cervical dislocation and their small intestines investigated to determine SFB

scores as described above. Light micrographs of mucosal smears were prepared by means of a Leitz Orthoplan photo microscope. Scanning electron micrographs of the ileal wall, 1 cm from the ileocecal junction, were made following the method described by Koopman et al. (1987). To confirm absence of bacteria other than SFB's in these mice, intestinal and cecal tissue and their contents and fecal samples of each mouse were cultured in broth (Koopman et al. 1983) for 24 h at 37°C and subsequently subcultured on enriched blood agar plates for 24 h at 37°C under either aerobic or anaerobic conditions. Light microscopic examination of cecal contents and of fecal samples of each mouse was performed to detect any noncultivable bacteria other than SFB's. Thirty days after inoculation, bacteriological examination of pooled samples of feces from the isolator was carried out to further exclude the presence of bacteria other than SFB's.

## Results

In order to induce colonization of germ-free mice by SFB's within 7 days, $2 \times 10^4$ SFB's had to be administered by the orogastric route and $2 \times 10^2$ by intraileal injection (Table 1). Thus, intraileal injection was more effective.

In experiment 2, all donor mice were found to be SFB-positive. SFB scores of these animals were $87 \pm 21$ (mean $\pm$ SD, $n = 10$) for the distal and $22 \pm 20$ for the proximal site of the intestinal section removed for preparation of the homogenate. The concentration of SFB's in the homogenate was $1.5 \times 10^7$/ml which is equivalent to $8.4 \times 10^7$/g intestinal tissue. Ethanol treatment and subsequent anaerobic incubation for 42 h did not change SFB concentration. After 42 h of incubation of the ethanol-treated, undiluted and diluted homogenates, bacterial growth was visible in the undiluted, 10-fold and $10^2$-fold diluted homogenates. There were Gram-positive, polymorphic, spore-forming bacteria as detected by light microscopy. The homogenates diluted $10^3$ or more did not contain bacteria other than SFB's after either anaerobic or aerobic incubation. The $10^4$-fold diluted homogenate was chosen for inoculation. The concentrated suspension prepared from this dilution contained $6.6 \times 10^4$ SFB's/ml, of which 0.1 ml was inoculated intraileally.

All cage mates of inoculated mice examined either 5, 10 or 15 days after inoculation were found to harbor SFB's. SFB scores were (mean $\pm$ SD, $n = 4$): after 5 days, $15 \pm 10$; after 10 days, $69 \pm 14$; after 15 days, $65 \pm 11$. The appearance of SFB's in mono-associated mice as seen in Gram-stained mucosal smears by means of which SFB scores were determined, is demonstrated in Fig. 1. As a comparison, SFB's adhering to the ileal mucosa as seen by the scanning electron microscope, are shown in Fig. 2. Aerobic and anaerobic culturing of small intestinal and cecal contents and tissues, and fecal samples did not show cultivable bacteria. By light microscopic examination of cecal contents and feces non-cultivable bacteria other than SFB's could not be detected. The same results were achieved by cultural and microscopic examination of feces, 30 days after inoculation.

## Discussion

Our method of isolating SFB's from mice with a microflora of SFB's and clostridia and administrating them to germ-free mice resulted in recipients mono-associated with SFB's. To our knowledge, mice colonized by SFB's only are now available for the first time. This may have important consequences for the progress in research on characteristics of SFB's such as their taxonomy and their impact on the host in terms of gastrointestinal physiology, colonization resistance and immunity.

The choice of the intraileal route for administration, which is very effective, enabled us to use the technique of dilution to eliminate other bacteria. Diluted homogenates free of detectable, undesirable bacteria still contain sufficient SFB's to induce colonization in germ-free mice. In a preliminary study (Klaasen et al. 1990b), intestinal homogenates of SPF mice were treated with both chloro-

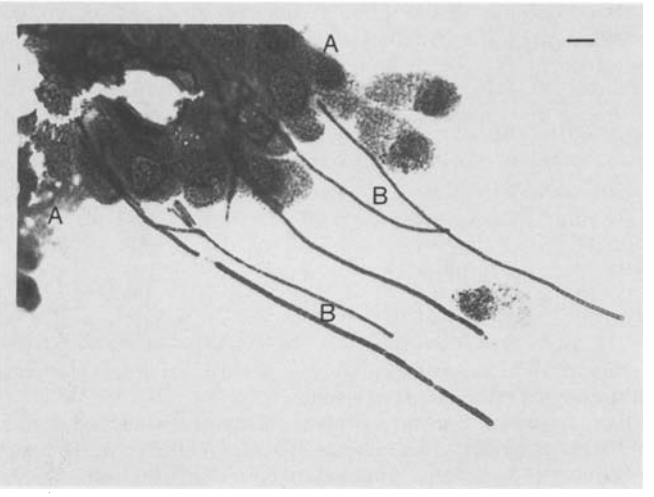


**Fig. 1A, B.** Segmented filamentous bacteria (SFB's) in a Gram-stained smear prepared from the ileal mucosa (1 cm from the ileocecal junction) of a 6-week-old mono-associated mouse. **A** Mucosal material mainly existing of epithelial cells and mucus. **B** SFB's with one end attached to the mucosa. Light micrography, 595×, bar = 6.0 μm

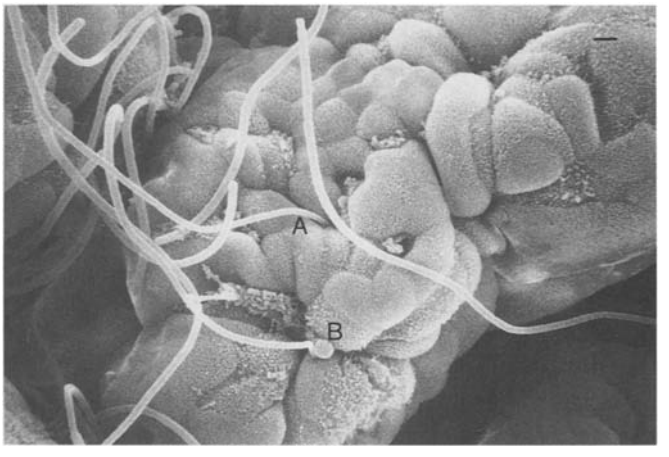


**Fig. 2A, B.** Segmented filamentous bacteria (SFB's) attached to the intestinal villus epithelium (1 cm from the ileocecal junction) of a 6-week-old mono-associated mouse. **A** SFB with one end firmly attached to the epithelium. **B** Lymphocyte. SEM, 859×, bar = 3.2 μm

form and ethanol to inactivate vegetative forms of bacteria. Administration of this homogenate to germ-free mice yielded animals associated with both SFB's and clostridia. It was unclear whether these clostridia originated from the SPF donor mice or had been present in the ethanol and/or chloroform. In any event, we now used ethanol which had been filtered. Chloroform, which damaged the filter, had to be omitted.

In order to produce mice mono-associated with SFB's, we used SFB donor mice which had a flora consisting of both SFB's and clostridia (Klaasen et al. 1990b), and the technique of intraileal administration. Possibly, when using the combination of treatment of intestinal homogenates with filtered ethanol, dilution and intraileal inoculation, SPF mice might be suitable as SFB donors. This would simplify the procedure to generate mice mono-associated with SFB's. Prerequisite might be that the SPF donor mice are heavily colonized by SFB's.

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