

## Growth factors for Nanobacteria

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### ABSTRACT

Nanobacteria are novel micro-organisms recently isolated from fetal bovine serum and blood of cows and humans. These coccoid, gram negative bacteria in alpha-2 subgroup of Proteobacteria grow slowly under mammalian cell culture conditions but not in common media for microbes. Now we have found two different kinds of culture supplement preparations that improve their growth and make them culturable in the classical sense. These are supernatant fractions of conditioned media obtained from 1-3 months old nanobacteria cultures and from about a 2 weeks old *Bacillus* species culture. Both improved multiplication and particle yields and the latter increased their resistance to gentamycin. Nanobacteria cultured with any of the methods shared similar immunological property, structure and protein pattern. The growth supporting factors were heat-stable and nondialyzable, and dialysis improved the growth promoting action. Nanobacteria formed stony colonies in a bacteriological medium supplemented with the growth factors. This is an implication that nanobacterial growth is influenced by pre-existing bacterial flora.

Keywords: Nanobacteria, microbial growth factor, symbiosis, calcification, apatite

### 1. INTRODUCTION

Culturability of an organism makes the microbiologist's life easier. Some micro-organisms cannot be cultured at all in laboratories either because the necessary conditions for their cultivation *in vitro* are not yet known or because the available culture systems are inefficient and impractical. The chemical composition of the habitat and, if existing, the other flora member(s) should be scrutinized for making an estimation for necessary needs for cultivating the isolate. Nanobacteria which can be cultured from serum share similarities with poorly characterized pseudo-organisms classified as artefacts,<sup>1,4</sup> but sometimes as L-forms<sup>5</sup> or mycoplasma-like organisms.<sup>6</sup> Nanobacteria differ strikingly from all well-characterized microbes. Due to the carbonate apatite layer, nanobacteria are very difficult to kill, break or fix, have very peculiar staining properties, and are also very resistant to antibiotics.<sup>7-12</sup> Sequences of nanobacteria proteins are unique and monoclonal antibodies produced against intact nanobacteria do not recognize any proteins in human sera or in 25 common bacterial species, including 9 mycoplasma species. Nanobacteria cause lysis of mammalian cells and their internalization by fibroblasts has been illustrated.<sup>13</sup> Our group has endeavoured this bacteria for 10 years,<sup>14</sup> and tentatively called them as *Nanobacterium sanguineum* and deposited in the German Collection of Micro-organisms (no. 5819-5821, Braunschweig, Germany). About a hundred batches of sterile fetal and newborn bovine sera have been tested by culture and most of them have been found contaminated by nanobacteria.<sup>8</sup> Because of their poor culturability, and unique properties, they remained as unidentified in the sterility control tests for many years.

#### 1.1. Symbiosis among bacteria:

All living organisms, many nonliving materials, and even air are used as habitats by other organisms. Many organisms live much of their lives in a special ecological relationship: an important part of their environment is a member of another species. Any micro-organism that spends a portion or all of its life physically associated with another organism of a different species is called a symbiont (or symbiote), and the relationship is designated as symbiosis. For example, a very important syntrophism occurs in the anaerobic methanogenic ecosystem where fatty acids are degraded to produce H<sub>2</sub> and methane by two different bacterial groups. In this ecosystem the production of the methane by methanogens depends on interspecies hydrogen transfer. One micro-organism (a fermentative bacterium) produces hydrogen gas, another micro-organism (a methanogen) uses it quickly as a substrate for methane gas production.<sup>15</sup>

#### 1.2. Microbial growth factor:

Some bacteria produce growth factor for itself or for each other. For example, viable cells of *Micrococcus luteus* secrete a factor, which promotes the resuscitation and growth of dormant, nongrowing cells of the organism. The resuscitation-promoting factor (Rpf) is a protein, which has been purified to homogeneity. In picomolar concentrations, it increases the viable cell count of dormant *M. luteus* cultures at least 100-fold and can also stimulate the growth of viable cells. Rpf also stimulates the growth of several other high G+C Gram positive bacteria including *Mycobacterium avium*, *Mycobacterium bovis* (BCG), *Mycobacterium kansasii*, *Mycobacterium*

*smegmatis*, and *Mycobacterium tuberculosis*.<sup>16</sup> An other example is a new microbial growth factor produced by a *Streptomyces* sp. Three soil bacteria show an absolute growth requirement for this growth factor and it is not found in the soil itself.<sup>17</sup>

### 1.3. Nanobacterial growth factor:

Nanobacteria are fastidious but culturable organisms. They were cultured in cell culture medium supplemented with L- glutamine and 10% FBS as a source of nanobacteria and as their supplement, at 37°C in atmosphere of 5% CO<sub>2</sub>-95% air typically for 3-4 weeks.<sup>13</sup> Increasing numbers of coccoid particles with a diameter of 0.08-0.5 µm, passing effectively through 0.2 µm filters, and showing typical doubling times of 1-5 days, could be detected. γ-irradiated serum gave negative culture results but supported the growth of nanobacteria subcultures.<sup>13,14</sup> Low yield, up to 3-month long culture time and attachment to the culture vessel made their harvesting difficult. Numerous amino acids, vitamins, salts, minerals, sugars and protein products have been tested without success to improve their growth. In this work, we tested conditioned medium preparations from bacteria, nanobacteria and mammalian cells as growth supplements and found two to enhance nanobacteria growth, i.e. growth factors. They were supernatant fractions of conditioned media from cultures of a *Bacillus* species and of nanobacteria (BGF and N3, respectively).

## 2. METHODS

BGF supplement was a sterile filtrate of a 14-days old *Bacillus* culture in RPMI-1640 without serum. The *Bacillus* providing the BGF was initially isolated from an exceptionally well-growing nanobacteria culture contaminated with common bacteria and did not possess any plasma coagulase activity. N3 supplement was the ultracentrifuged (100000g for 1.5 h), sterile-filtered (0.2 µm) supernatant fraction of a 1-3-months old nanobacteria culture in RPMI-1640 supplemented with 10% FBS. Sterile filtration was performed twice, the second time after one week of storage at 4°C (essential step). Both BGF and N3 were confirmed to be free of nanobacteria and other cultivable micro-organisms by classical microbiology culture techniques.

Cultures in 96-well plates (100 µl amounts) were incubated at 37°C under an atmosphere of 5% CO<sub>2</sub>- 95% air at 90% humidity. Optical densities (OD) at 650 nm were followed with an ELISA reader in cultures started by adding commercial fetal bovine serum (FBS; Sera-Lab, batch no 901046) at 10% concentration to DMEM, without (C.C.) or with 1 mg/ml gentamycin (+G), supplemented with 10% *Bacillus* conditioned medium (+BGF) or 10% nanobacteria conditioned medium (+N3). FBS functioned as the source of the nanobacteria and also as a necessary part of the medium. Each value is the mean ± standard deviation of 26-32 incubations. Culture with γ-irradiated (3 megarad) Sera-Lab FBS with N3 served as a negative control.

Subcultures were started at day 20 from cultures similar as described above. After splitting 1: 100 with fresh medium omitting growth factor preparations (with or without gentamycin) containing nanobacteria-negative serum (a commercial FBS batch tested to be free of nanobacteria), culturing was continued in 96-well plates. As a control, culture with negative serum ± BGF was used.

TEM and SEM samples were prepared as described previously<sup>13</sup> except that the fixative was 4% formaldehyde- 0.1 % glutaraldehyde.

Cultures on solid media were established using Loeffler medium supplemented with 10% BGF and DMEM replaced water in the formula<sup>18</sup> and were inoculated with 2-week old nanobacteria culture (30µl/10 cm plate). Cultures were performed at 37°C under an atmosphere of 5% CO<sub>2</sub>-95% air in a humidified chamber for 10-14 days.

## 3. RESULTS

As seen in Fig. 1, BGF shortened the lag phase, improved the growth rate and the final yield of nanobacteria. With N3, the results were between those of BGF and the control (C.C.). Culture with γ-irradiated serum was negative (Fig. 1a). Gentamycin, at 1 mg/ml, inhibited nanobacteria growth (Fig. 1b).

Nanobacteria cultures with the growth supplements resulted in a yield of wet pellet of approximately 2-4 µl/ml in two weeks. Similar yields could be obtained without supplements only after 1-3 months. A wet pellet of 2 µl/ml is equivalent to OD<sub>650</sub> 0.05 in 96-well plates having 200 µl cultures and this yield was obtained in the experiment shown in Fig. 1a, Optimal nanobacteria

growth was obtained with 10-20% BGF while 50% proved toxic. BGF from 1-week old culture was inferior to that from 2-week old culture and BGF from 1-month old culture was toxic.

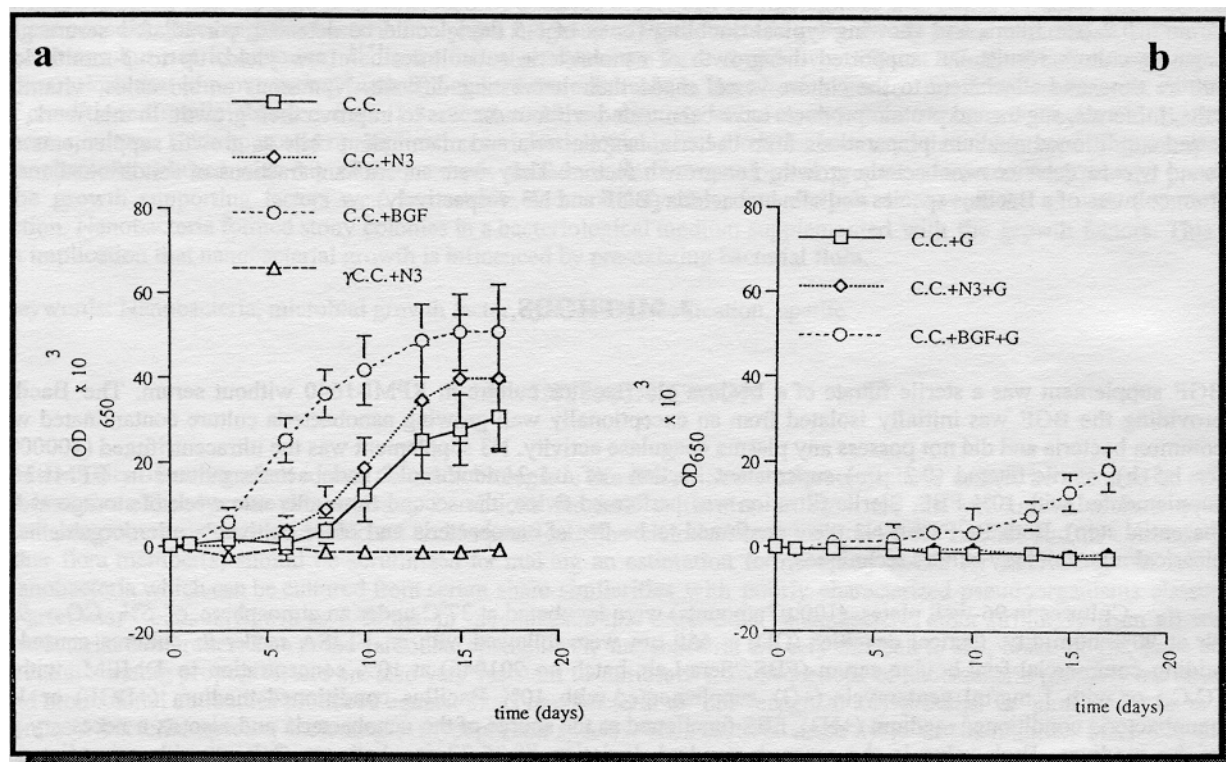


Fig. 1. Growth curves of nanobacteria with or without conditioned media in the absence (a) and presence of 1 mg/ml gentamycin (b).

Best N3 preparations were obtained from 1-3-month old nanobacteria cultured in RPMI-1640 medium supplemented with 10% FBS. N3 was effective at 10-90% concentration. Combining N3 to BGF gave the same growth as BGF alone. Dialyzed N3 enhanced the growth as well as dialyzed BGF ( $OD_{650}$  C.C.+N3  $0.056 \pm 0.001$ , C.C. +BGF  $0.060 \pm 0.008$ ,  $n=4$ ,  $t=1$  week, compare to Fig. 1a). Thus the growth supporting factors were nondialyzable. The factors were heat-stable because boiling them for 30 min did not change the results.

Nanobacteria passaged in liquid culture grew in subcultures (omitting growth factor preparates) without a lag period and slightly faster than in the primary culture (Fig. 2a). As a control, culture with negative serum  $\pm$  BGF was used. The apparent increase in the  $OD_{650}$  of negative controls and gentamycin containing cultures after day 10 are artifactual due to slow evaporation of liquid resulting in more concentrated medium in the culture wells. Each point in

the figures represents the mean  $\pm$  S.D. of 6-8 cultures. Subcultures of C.C.+G (not shown) were similar to those with N3+G.

When the curves were redrawn with OD on log scale (not shown), C.C. and C.C.+N3 showed logarithmic growth for 3-4 doublings between days 6-10 with a doubling time of approximately 1 day. With BGF the growth was faster, doubling time was less than 0.6 day at the shortest and more than 6 doublings took place. Interestingly, C.C.+BGF+G showed logarithmic growth between days 3-17 with a doubling time of 3 days.

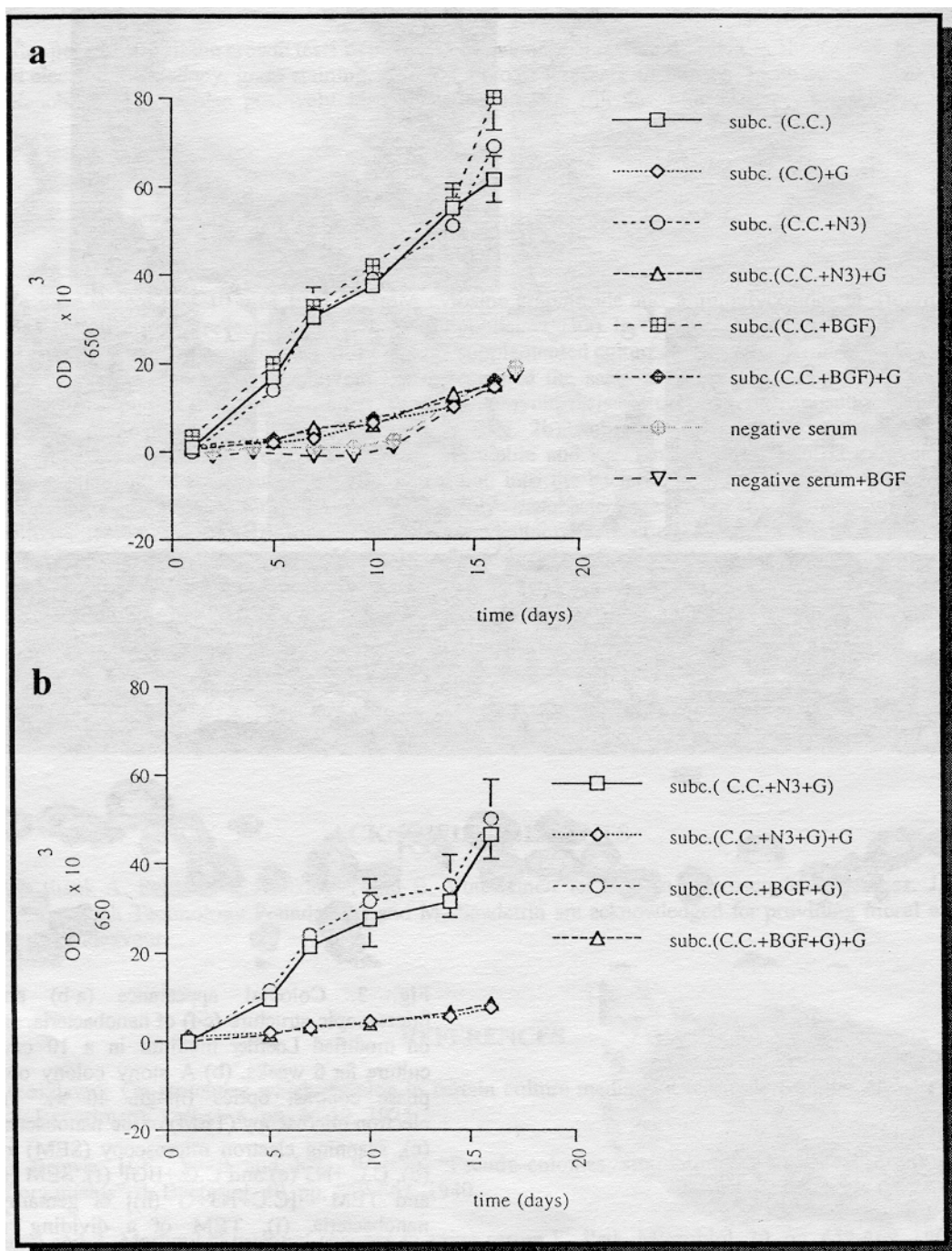


Fig. 2. Subcultures of nanobacteria from Fig. 1a (a) and 1b (b) with and without gentamycin.

With the growth factors, nanobacteria could be cultured on solid microbiological medium. Approximately 1 mm sized colonies with smooth surfaces reached through the Loeffler medium layer and attached to the bottom (Fig.3 a and b).

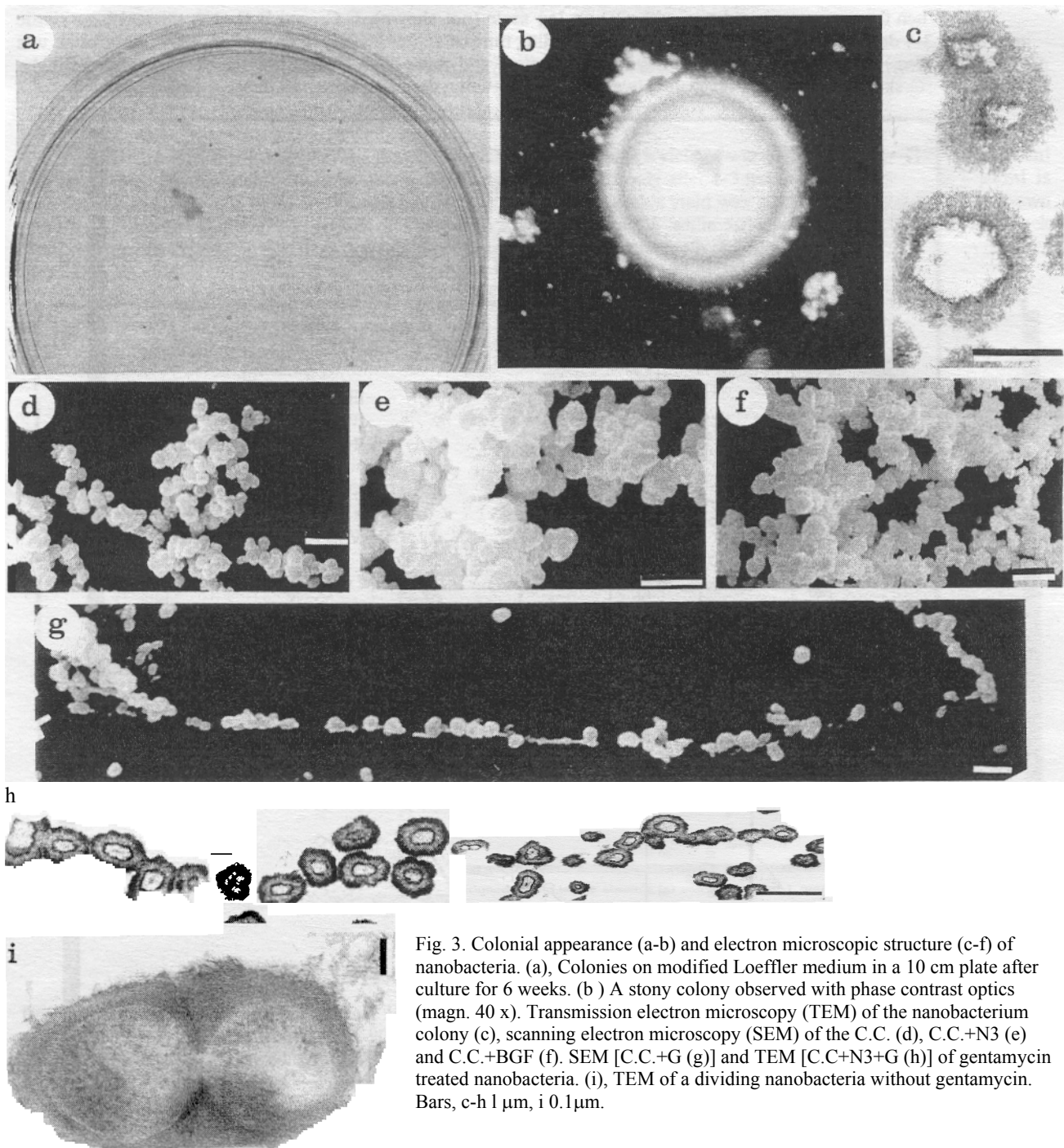


Fig. 3. Colonial appearance (a-b) and electron microscopic structure (c-f) of nanobacteria. (a), Colonies on modified Loeffler medium in a 10 cm plate after culture for 6 weeks. (b) A stony colony observed with phase contrast optics (magn. 40 x). Transmission electron microscopy (TEM) of the nanobacterium colony (c), scanning electron microscopy (SEM) of the C.C. (d), C.C.+N3 (e) and C.C.+BGF (f). SEM [C.C.+G (g)] and TEM [C.C.+N3+G (h)] of gentamycin treated nanobacteria. (i), TEM of a dividing nanobacteria without gentamycin. Bars, c-h 1  $\mu$ m, i 0.1  $\mu$ m.

Subculture of the colony to nanobacteria culture medium and bacteriological stainings revealed only nanobacteria as also illustrated with transmission electron microscopy (TEM) of the colony (Fig. 3c). Scanning electron microscopy (SEM) of the C.C. (d), C.C.+N3 (e) and C.C.+BGF (f) revealed similar morphology. SEM of all samples with gentamycin showed also similar particles attached to each other by rope-like filaments as seen for C.C.+G (Fig. 3g). TEM revealed the same appearance and showed capsule-like structures as seen for C.C.+N3+G (Fig. 3h), and (i) shows TEM of a dividing nanobacteria without gentamycin. Nanobacteria cultured with either N3 or BGF appeared as non-adherent typical coccoid particles sometimes in clumps. In control cultures, similar particles were in larger clumps often adhering to the culture vessel (Fig. 3d-f).

After performing all the growth tests depicted, only nanobacteria were detected in the culture media by phase contrast and electron microscopy, gram staining, and bacteriological culture in nutrient broth and on sheep-blood agar medium. Nanobacteria were also positively identified with ELISA and dot blot assays with specific monoclonal antibodies.

#### 4. DISCUSSION

We have shown that 10 mM EDTA, 1mM cytosine arabinoside and aminoglycosides at 1mg/ml can stop nanobacteria multiplication as revealed by ELISA (our unpublished data). In this work, 1mg/ml gentamycin effectively blocked the growth of nanobacteria in the control and N3 supplemented cultures but cultures with BGF multiplied after a lag period of 3 days (Fig. 1b). Gentamycin levels remained the same in all cultures as tested with minimum inhibitory concentration test by using *B. subtilis*. When gentamycin resistant cultures were subcultured without BGF, they did not grow in the presence of 1 mg/ml gentamycin (Fig. 2b ). Subcultures made from nongrowing gentamycin- supplemented control and N3-cultures after 20 days were viable and grew in the absence of gentamycin (Fig. 2b ). Resistance to gentamycin may be caused by poor penetration into the bacteria, poor affinity for ribosomal subunits or by enzymatic degradation of aminoglycosides.<sup>19</sup> Possibly nanobacteria resistance to gentamycin was related to impermeability due to their thick capsule-like structure. Gentamycin exerted only a bacteriostatic action against nanobacteria even at concentrations comparable to those found in urine of patients treated with gentamycin.

With the aid of the new growth-factors, nanobacteria could be the first time cultivated on solid microbial media (Fig. 3a-b) and revealed substantial passageability while retaining their unique features, e.g. size, shape, cell-wall structure and division mode (Fig 3c-f). It is remarkable, and also alarming considering possible pathogenicity, that nanobacteria produce growth factor(s) for their own needs and can also exploit factors released by at least some *Bacillus* species. Although the identity of these factors awaits for clarification, they are highly beneficial in raising nanobacteria in amounts needed to solve their unique structures and place in the order of living creatures.

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