Extraordinary survival of nanobacteria under extreme conditions

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ABSTRACT

Nanobacteria show high resistance to gamma irradiation. To further examine their survival in extreme conditions several disinfecting and sterilizating chemica1s as well as autoclaving, UV light, microwaves, heating and drying treatments were carried out. The effect of antibiotics used in cell culture were a1so eva1uated. Two forms of nanobacteria were used in the tests: nanobacteria cultured in serum containing medium, and nanobacteria cu1tured in serum-free medium, the latter being more minera1ized. Nanobacteria, having various amounts of apatite on their surfaces, were used to ana1yze the degree of protection given by the minera1.

The chemica1s tested included ethanol, glutara1dehyde, fonna1in, hypochlorite, hydrogen peroxide, hydrochloric acid, sodium hydroxide, detergents, and commercia1 disinfectants at concentrations genera1ly used for disinfection. After chemical and physical treatments for various times, the nanobacteria were subcultured to detect their survival. The results show unique and wide resistance of nanobacteria to common agents used in disinfection. It can a1so be seen that the mineralization of the nanobacteria1 surface furthennore increases the resistance. Survival of nanobacteria is unique among living bacteria, but it can be compared with that observed in spores. Interestingly, nanobacteria have metabolic rate as slow as bacteria1 spores. A slow metabolic rate and protective structures, like minera1, biofilm and impermeable cell wa1l, can thus explain the observations made.

Keywords: nanobacteria, disinfection, sterilization, heat resistance, radiation resistance

1. INTRODUCTION

Competition for nutrients necessary for life is enormous in natura1 environments and thus clever adaptations and surviva1 strategies for unfavorable conditions are needed. Bacteria can form spores, cysts and biofilm, which help them survive unfavorable periods of time. Bacteria in such forms have lead to significantly slower metabolic functions, but a1so vegetative cells can slow down their metabolism.[1] The increased resistance of bacteria in biofilm or as spores is not only because of the slower metabolic rate. The impermeable structures around the organism serve as mechanica1 barriers blocking the entrance of potentially harmful compounds. Some additiona1 mechanisms are also known which help in the surviva1 of bacteria. The heat resistance of bacterial spores can be attributed to three main factors, these are protoplast dehydration, mineralization and thermal adaptation.[2] Radiation resistance is commonly associated with sophisticated DNA repairment systems. Minimizing metabolic rate and multiplication are obviously the main preconditions for bacterial survival, allowing time for the repairment of DNA and other damaged cellular components. Very slow metabolism, and ability to form biofilm are also characteristics of nanobacteria. Because of the minima1istic size, the presence of complicated systems for nucleic acid repairment in nanobacteria seems very unlikely. A possible explanation for the observed gamma irradiation resistance may be the very small size and the peculiarities in the nucleic acid structure. (see Ref. 3)

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To examine the survival of nanobacteria in extreme conditions. severa1 chemica1 and physical treatments were carried out. The selection of an appropriate test for disinfection is not straightforward. and accurate comparisons of the results obtained from different tests are often impossible. This is due to the several factors which affect the disinfection result. These factors include duration of exposure. presence of organic load, type. age, concentration and diluent of the disinfectant. and number. age. growth form of the micro-organisms present. and the temperature. Current1y there are severa1 types of disinfection tests. but these are mainly suitable for rapidly growing bacteria. The disinfection tests of slowly growing Mycobacteria, some of which are extremely resistant. have long suffered from lack of appropriate. reliable standardization.[4] Typica11y. centrifugation or very high dilution have been used to eliminate the effect of residua1 concentrations of disinfectants. Subsequent1y. plating on agar medium for colony count is done for eva1uating the reduction in viability. For nanobacteria such assays are not suitable. Recovery of nanobacteria by centrifugation generally results in unpredictable losses. Due to their slow growth rate. high dilutions result in very long incubation times. and extremely poor culturability on solid media makes the evaluation of a nanobacteria count impossible.

Mineralization is the most characteristic property of nanobacteria, and possibly the main mechanism of pathology caused by the organism.[5] The mineral formed under standard culture conditions is hydroxyl or carbonate apatite as revealed by several methods, including energy dispersive X-ray microanalysis and Fourier transform IR spectroscopy (Kajander *et al.,* in this issue). One of the primary functions of the mineral may be protection against harsh environmental conditions. The apatite can prevent the penetration of harmful compounds to the interior of the organism. Depending on the culture time and culture conditions, various degrees of mineralization has been observed. Mineralization by nanobacteria cultured without serum (SF-nanobacteria) is much more extensive than that observed in nanobacteria cultured with serum containing medium.[6] The doubling time of serum nanobacteria and SF-nanobacteria, are about three days and six days respectively, measured by amino acid incorporation.[6]

Disinfecting chemica1s at concentrations genera11y used have now been tested against cultured nanobacteria. The chemica1s selected represent a wide variety of mechanisms which are known to affect biologica1 systems. Surviva1 of nanobacteria at high temperature, in drying and under UV-C irradiation was also tested. There are severa1 mechanisms for antibiotic resistance in bacteria which have not been discussed here. We eva1uated the effect of four antibiotics against nanobacteria. The antibiotics are those commonly used in cell culture.

2. EXPERIMENTAL DESIGN

2.1 NANOBACTERIA CULTURE IN SERUM CONTAINING MEDIUM

Nanobacteria were cu1tured with 10% fetal bovine serum in DMEM medium (serum nanobacteria) for one month at 37°C in an atmosphere of 5% CO_2 -95% air. The cu1tures were harvested by centrifugation. For the autoclaving. UV .microwave. heating and drying treatments, the harvested nanobacteria were suspended in phosphate buffered saline (pH 7.4; PBS). After treatments. subcu1turing of the nanobacteria was made in 10% gamma irradiated fetal bovine serum in DMEM medium. The growth of serum nanobacteria was fo1lowed by 1ight microscopy and absorbance measurement with a spectrophotometer at 650 nm.

2.2. NANOBACTERIA CULTURE WITHOUT SERUM

SF-nanobacteria were cultured in DMEM medium for one week at 37°C in an atmosphere of 5% CO_2 -95% air, and all the cultures firmly adhered to the culture vessel. The cultures were exposed to the disinfectants after removal of the culture medium. For the autoclaving, UV, microwave, and drying treatments, the medium was removed and an equa1 amount of PBS used instead. For the heat treatments, the SF-nanobacteria were harvested by scraping the culture vessel followed by centrifugation of the medium. The obtained pellet was suspended in PBS and used in the test. After treatments the SF- nanobacteria were subcultured in DMEM medium and the growth followed by light microscopy to see the adherence and typical mineralization

2.3 CHEMICAL DISINFECTION FOR SF-NANOBACTERIA

The concentrations of the chemica1s used were those commonly used for disinfection or as instructed by the manufacturer. The chemica1s included 70% ethanol, 2% glutaraldehyde, 4% formaldehyde, 0.5% hypochlorite, 3% hydrogen perox.ide, 1M hydrochloric acid (HCI), 1M sodium hydroxide (NaOH), 1% sodium dodecyl su1fate (SDS), 1% Tween 80, 1% Triton X-l00, 3M guanidium-hydroch1oride, 3M urea, 1% Virkon® (100% product contains 50% potassium persu1fate, 5% su1faminoic acid) (Antec Intemationa1 Ltd., Suffolk, England), 1.5% Erifenol® (100% product contains <5% NaOH, <5% o-benzyl-p- chlorophenol, 5-15% p-ch1oro-m-cresol)(Orion Oy, Finland), 1% Klorilli® (100% product contains sodium metasilicate, sodium N-chloro-p-toluenesulfonamide-3-hydrate and 20 000 ppm active chlorine) (Orion Oy, Finland), and 3% Buraton® (100% product contains 4.5% formaldehyde, 6.8% glyoxal, 1.5% glyoxylic acid, 6% dimethylaurylbenzylammonium chloride) (Schülke & Mayr, Germany). The dilutions to be used were freshly prepared on the day of exposure in sterile distilled water. As a positive control, only diluent was used. Negative control contained only culture medium.

The SF-nanobacteria were exposed to the chemicals for 10 and 30 minutes at room temperature after removal of the cu1ture medium. After exposure, the disinfectant solution was removed and fresh medium added (with a neutralization step in the case of HCl and NaOH). If any significant deattachment occurred, nanobacteria were recovered by centrifugation, and subcultured. The exposed serum-free cultures were passaged 1:10 after 48 hours and the growth was followed by light microscopy for three weeks.

2.4 AUTOCLAVING, UV, AND DRYING TREATMENTS

Serum and SF-nanobacteria were autoclaved in a sma1l volume of phosphate buffered saline (PBS), pH 7.4 at 121°C for 20 minutes. UV treatment was given to both nanobacteria in PBS in a laminar hood under Philips 15 W UV-C lamp for periods of 1 and 3 hours and overnightly in petri dishes with the lids removed. The distance of the cu1tures from the lamp was about 60 cm. Drying treatments were carried out by drying nanobacteria overnightly at room temperature or by heating for one hour at l00°C. SF-nanobacteria was dried only overnightly at room temperature. Microwave treatment was given by bringing the samples ten times to boiling point (100°C) in a 1400W microwave oven.

2.5 HEATING OF NANOBACTERIA

Heat effect on surviva1 of the nanobacteria was determined by exposing nanobacteria as pellets in PBS for 15 and 30 minutes, with temperatures varying between 60°C and 100°C. Exposed SF-nanobacteria were cu1tured in DMEM medium and the growth followed by microscopy as above. The growth of serum nanobacteria cultures was followed by light microscopy and absorbance measurement with a spectrophotometer at 650 nm.

2.6 ANTIBIOTIC SENSITIVITY TESTS

Antibiotic sensitivity of serum nanobacteria was tested with a mixture of penicillin (β-1actam) and streptomycin (aminoglycoside) (PS) at 1x and 10x concentration (100 IU penicillin, 100 µg/ml streptomycin = 1x), kanamycin (aminoglycoside) at 1x and 10x concentration (100 µg/ml = lx) and gentamycin (aminoglycoside) at 1x concentration (100 µg/ml). The 1x concentrations are those recommended for cell culture. After 10 days culture in 10% serum containing DMEM with the antibiotic, growth was compared to that of nanobacteria cultures without antibiotics present.

3.RESULTS

3.1 CHEMICAL DISINFECTION

SF-nanobacteria showed a wide resistance to the disinfectants used. Only Virkon was effective in killing SF-nanobacteria after thirty minutes. Hydrochloric acid treatment dissolved the apatite layer of nanobacteria, but remineralization was observed after addition of culture medium. The guanidium-hydrochloride and Buraton treatments resulted in the deattachment of the SF-nanobacteria, but the disinfection efficacy of Buraton was slightly less than that of guanidium-hydrochloride. Results of the chemica1 treatments are presented in Table 1.

	Exposure time	
Chemical	10 min	30 min
70% Ethanol	+++	+++
2% Glutaraldehyde	+++	+++
4% Formaldehyde	+++	+++
0.5% Hypochlorite	+++	+++
3% H_2O_2	+++	+++
1M HCl	n.d.	++ *
1M NaOH	+++	+++
1% SDS	+++	+++
1% Tween 80	+++	+++
1% Triton X-100	+++	+++
3M Guanidium-HCl	n.d.	+ *
3M Urea	+++	+++
1% Virkon®	n.d.	- *
1.5% Erifenol®	+++	+++
1% Klorilli®	+++	+++
3% Buraton®	n.d.	++ *

* = partial or total deattachment on exposure
n.d. = not determined

Table I. Resistance of SF-nanobacteria to chemical disinfectants. Survival was determined after subculture by comparison to the treatment with onlv diluent.

+++: No effect; ++: Reduced survival; +: Markedly reduced survival; -: **No survival**

3.2 AUTOCLAVING, UV, AND DRYING TREATMENTS

Drying at a temperature of 100°C kil1ed serum nanobacteria, but drying at room temperature did not. Autoclaving was not detrimental to the SF-nanobacteria, but a marked reduction in the survival of serum nanobacteria was observed. SF- nanobacteria tolerated UV light with no effect on growth, but serum nanobacteria was significantly inactivated. Nanobacteria samples dried during the overnight UV treatment, and thus there became an additional stress for the organisms. Drying obviously had little or no effect to the result, since the survival of nanobacteria with all the UV treatments was similar. Because of lack of an UV radiometer, no UV dosage could be calculated, and more accurate tests with nanobacteria in culture medium should be conducted. Microwave treatment was more like a heat shock treatment than a sterilization step, short boilings being completely ineffective. Results of the follow-up of the nanobacteria survival after autoclaving, UV , microwave and drying treatments are presented in Table 2.

Table 2. Survival of nanobacteria after physical exposures. SF-nanobacteria was much more resistant than nanobacteria cultured with serum. SF-nanobacteria survived all test conditions without a marked reduction in viability. Serum nanobacteria were killed by drying for one hour at 100°C, and survival was markedly reduced in all other test conditions.

Treatment	Survival of serum nanobacteria	Survival of serum free nanobacteria
Autoclave	+	+++
UV irradiation 1h	+	+++
UV irradiation 3h	+	+++
UV irradiation overnightly	+	+++
Microwaves	+++	+++
Drying in RT	+	+++
Drying at 100°C	-	n.d.

3.3 HEAT RESISTANCE OF NANOBACTERIA

Nanobacteria were very heat resistant. Fifteen minutes boiling was not enough for killing serum nanobacteria, but thirty minutes inactivated them. Growth curves of serum nanobacteria after heat treatment are presented in Figure I. Importantly. the growth of serum nanobacteria was very similar. with no observed lag period. even after the fifteen minute boiling. Microscopica1 observations of the SF-nanobacteria cultures after heat treatment revea1ed that they had survived all the tested conditions including boiling at 100°C for 30 minutes. Initially, reduction in the amount of viable SF-nanobacteria was observed with the higher temperatures, but after two weeks there was no difference in the test culture resu1ts as compared to the non-heated control.

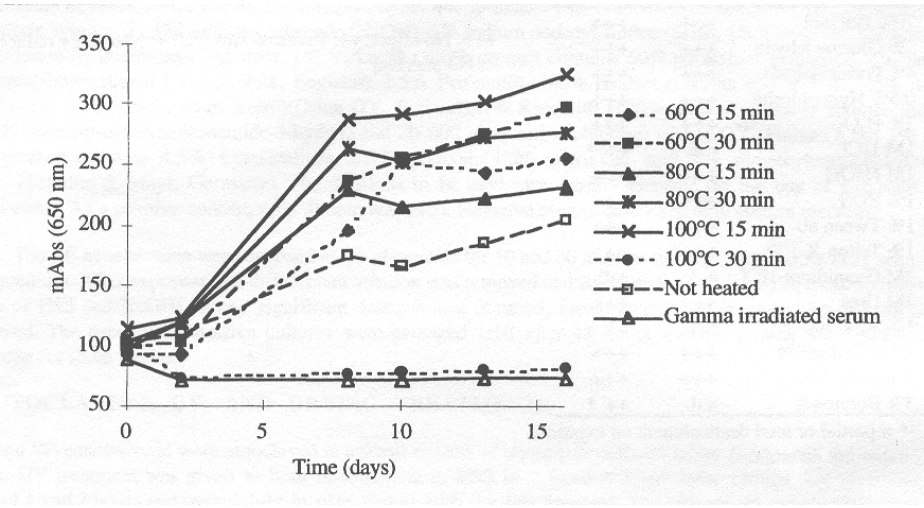

Figure 1. Graphic showing the effect of heat on the growth of serum nanobacteria. Nanobacteria were exposed for heat in PBS and cultured for 16 days. Only thirty minutes boiling resu1ted in the inactivation of nanobacteria. Exponential growth was observed with all other treatments. The medium containing 10% gamma irradiated serum (Negative control) did not show any grow.

3.4 ANTIBIOTIC RESISTANCE OF NANOBACTERIA

High resistance to the tested antibiotics was observed. Ten times higher concentrations than normally used in ce11 culture were needed to prevent the growth of nanobacteria. Figure 2 shows the effect of antibiotics on growth of nanobacteria cultured with serum containing medium. Interestingly, at concentrations of antibiotics with no effect on growth, there was a profound effect in the morphology of nanobacteria as seen in SEM (Figures 3A and 3B). This suggests that nanobacteria have adaptive ways for protecting themselves for detrimental attacs, e.g., by secreting slimy layers.

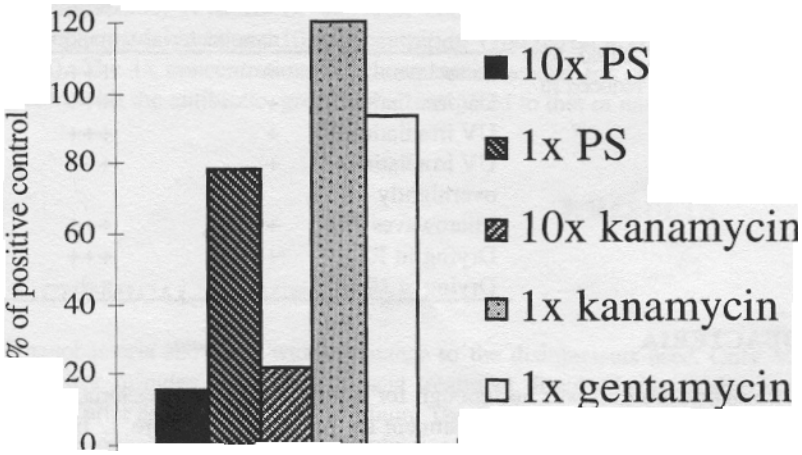

Figure 2. Graphic presenting the effect of antibiotics on nanobacteria1 growth. The growth is compared to that of nanobacteria cultured without antibiotics. Doses of antibiotics ten times higher than recommended for use in ce11 culture were needed to prevent the growth of nanobacteria.

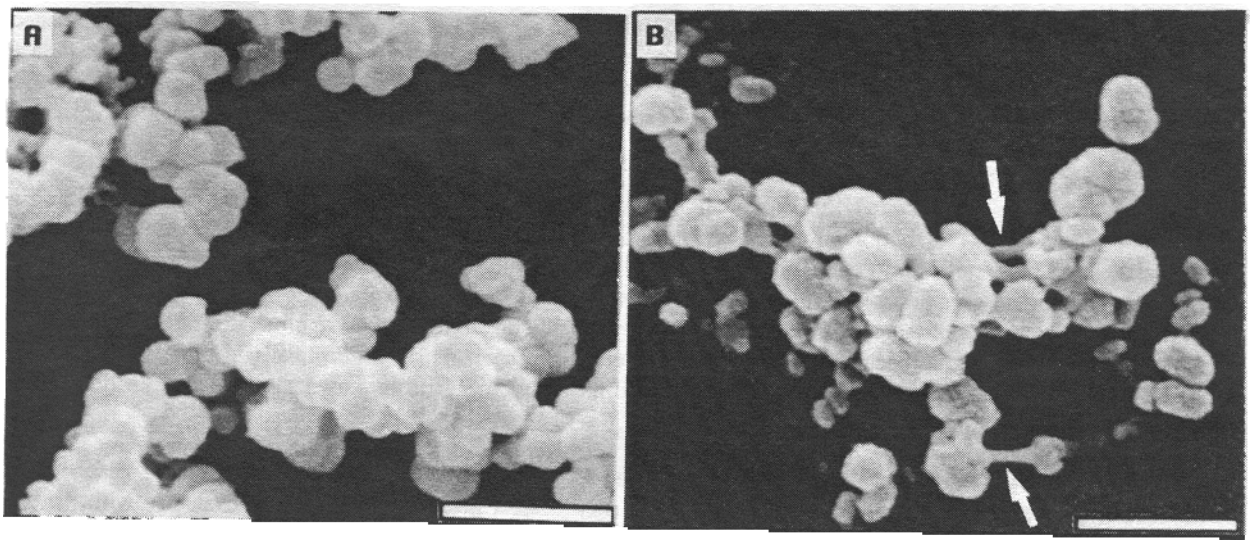

Figure 3. SEM images of nanobacteria cultured with and without antibiotics for one month in medium containing 10% FBS. Bars 1 μm. (A) Nanobacteria cu1tured without antibiotics. (B) Nanobacteria cultured with 100 μg/m1 gentamycin. Arrows show changes in the morphology.

4. CONCLUSIONS

Nanobacteria can tolerate harsh conditions extremely well. SF-nanobacteria were much more resistant than the nanobacteria cultured in serum containing medium. Extremes in pH, oxidizing agents, free chlorine, and chemica1s affecting the proteins as well as irradiation, heat and drying have very little effect on SF-nanobacteria. This indicates that the minera1 layer offers extra protection to the organism. Exceptiona1 surviva1 of nanobacteria has a1so been observed in association with human kidney stones. Viable nanobacteria were recovered from a1most all kidney stones by demineralizating the stones with hydrocloric acid (Ciftcioglu, Björklund, and Kajander, in this issue).

An effective way to eradicate nanobacteria with disinfecting chemicals, should include a demineralization step. Apatite can be dissolved at low pH or by means of calcium chelators such as ethylenediaminetetraacetic acid (EDTA). A second step should be then included to kill the organism by another mechanism. Virkon, composed of peroxygen compounds, surfactant, organic acids and an inorganic buffer system, proved to be effective against nanobacteria most likely because of the acidity (1% solution in water has pH 2.6) combined with other disinfection mechanisms.

Doses of three megarads gamma irradiation are needed to ensure destruction of nanobacteria[3]. Gamma irradiation is probably the best and most reliable method for killing nanobacteria. Drying at elevated temperatures or boiling for extended periods, can also be used in eradicating nanobacteria. Boiling for 30 minutes is effective against almost all living organisms, except some endospores, especially the spores of *Bacillus stearothennophilus*[2] and hyperthermophilic archae having 90°C or more as optimum temperature for growth.[7] This treatment is also not enough to kill SF-nanobacteria. Importantly, the normal autoclaving procedure (121°C for 20 min) was also inefficient in eradicating nanobacteria. Tests with higher temperatures and longer times should thus be conducted to determine an optimal procedure for the sterilization of nanobacteria contaminated equipment. The results indicate that nanobacteria represents a big sterility threat.

Resistance of nanobacteria to the tested antibiotics was very high. Cell culture antibiotics used in this study are effective only in very high concentrations. A possible resistance mechanism is the production of a protective slime as revealed by SEM. Modificating the cell wall is a common strategy for many bacteria to acquire resistance to antibiotics. As nanobacteria faces unfavorable conditions it starts to secrete polymers and form mineral upon them. Unfortunately SEM work with the other treatments was not conducted, and thus it is now impossible to say if similar changes are seen also with the disinfectants. The tested antibiotics were mainly aminoglycosides. A wider spectrum of antibiotics with different mechanisms of action should be tested.

Because different test methods. direct comparisons with other bacteria or spores cannot be made. Especially. methods for quantitation of nanobacteria would be needed to assess this. Observed resistance of serum nanobacteria shows that it is at least as resistant as Mycobacteria[4] and *Bacillus subtilis* spores.[8] which are the model organisms for disinfection resistance. The resistance of SF-nanobacteria is clearly superior to these. but comparative studies in identica1 conditions should be made to confirm this.

The apatite mineral around the organism serves as a primary defense shield against various chemica1s and irradiation. The surviva1 is clearly not only due to the mineral, because treatment with 1M hydrochloric acid could not kill nanobacteria, and remineralization could be observed later in the culture. A double defense with the apatite layer and impermeable membrane combined with a very slow metabolism is a likely explanation for the observed resistance of nanobacteria. The increased resistance of SF-nanobacteria is probably due to the extensive mineralization, slower metabolism and adherence to surfaces. It has been observed that the resistance mechanisms are multiplicative,[9] thus nanobacteria having an apatite coat, impermeable cell wall, slow metabolism and possibly other stil1 unknown mechanisms, becomes extremely resistant to most disinfecting methods.

Novel methodology for the detection of life could be useful in assessing if a sample contains live nanobacteria after sterilization steps. On the other hand, the function of these methods for detecting life could be evaluated with nanobacteria having very slow metabolism and long doubling times.

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