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Effect of calcium on the surfactant tolerance of a fluoranthene degrading bacterium

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Abstract

Surfactants are known to increase the apparent aqueous solubility of polycyclic aromatic hydrocarbons (PAHs) and may thus be used to enhance the bioavailability and thereby to stimulate the biodegradation of these hydrophobic compounds. However, surfactants may in some cases reduce or inhibit biodegradation because of toxicity to the bacteria. In this study, toxicity of surfactants on *Sphingomonas paucimobilis* strain EPA505 and the effect on fluoranthene mineralization were investigated using Triton X-100 as model surfactant. The data showed that amendment with 0.48 mM (0.3 g l^{-1}) of Triton X-100 completely inhibited fluoranthene and glucose mineralization and reduced cell culturability by 100% in 24 h. Electron micrographs indicate that Triton X-100 adversely affects the functioning of the cytoplasmic membrane. However, in the presence of 4.13 mM Ca^{2+}-ions, Triton X-100 more than doubled the maximum fluoranthene mineralization rate and cell culturability was reduced by only 10%. In liquid cultures divalent ions, Ca^{2+} in particular and Mg^{2+} to a lesser extent, were thus shown to be essential for the surfactant-enhanced biodegradation of fluoranthene. Most likely the Ca^{2+}-ions stabilized the cell membrane, making the cell less sensitive to Triton X-100. This is the first report on a specific factor which is important for successful surfactant-enhanced biodegradation of PAHs.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of pollutants which due to both natural and man-made processes are widespread in the environment (Sims & Overcash 1983). The PAHs are considered hazardous because of cytotoxic, mutagenic, and carcinogenic effects (Sims & Overcash 1983, Cerniglia & Heitkamp 1989). The fate of these compounds in the environment and the remediation of PAH-contaminated sites are, therefore, of high public interest.

One of the major problems currently limiting technology development for bioremediation of PAHs is the issue of bioavailability (Wilson & Jones 1993). Due to the highly hydrophobic nature of PAH-molecules, the bacterial access to these sorbed and non-aqueous phase PAHs is limited (Cerniglia 1992, Harms & Zehnder 1995, Wilson & Jones 1993, Weissenfels et al. 1992). Surfactants applied at concentrations above the critical micelle concentration (CMC) are known to enhance the mobility and apparent solubility of hydrophobic compounds (Vigon & Rubin 1989, Liu et al. 1991, Edward et al. 1991). Therefore, use of surfactants has been proposed as a mechanism to enhance the bioavailability of hydrophobic pollutants for microbial degradation (Thiem 1994, Guerin and Jones, 1988). However, conflicting results regarding the stimulating effect of surfactants in biodegradation processes have been reported (Rouse et al. 1994). Successful surfactant-enhanced bioremediation is dependent on the bacterial tolerance to the surfactant. Therefore, knowledge on how a surfactant affects the survival and activity of the microbial degraders is essential.

Several mechanisms have been suggested to be responsible for the observed surfactant-induced reduction on PAH-mineralization. These include decreased

bioavailability of micelle-solubilized substrate (Laha & Luthy 1991; Thibault et al. 1996; Volkering et al. 1995; Guha & Jaffé 1996; Grimberg et al. 1995), disruption and/or permeabilization of cell membranes, or surfactant interactions with enzymes or proteins essential to the proper functioning of the cells (Laha & Luthy 1991; Thibault et al. 1996; Guerin & Jones 1988; Tsomides et al. 1995; Volkering et al. 1995; Rock & Alexander 1995; Liu et al. 1995). Neither of the above reports, however, have investigated the suggested bacterium-surfactant interactions in detail.

Amendment with the non-ionic surfactant, Triton X-100, has previously been shown to stimulate the mineralization of fluoranthene by *Sphingomonas paucimobilis* when grown in Bushnell-Haas medium (Lantz et al. 1995). However, our experimental work with the strain has demonstrated that it may be highly sensitive to Triton X-100 if grown in other media. We have, therefore, looked for specific components which could be responsible for this difference in tolerance to Triton X-100. Our data show that presence of Ca^{2+}-ions may greatly enhance the tolerance of *S. paucimobilis*, to Triton X-100 which in turn results in an increased degradation of fluoranthene. To the best of our knowledge this is the first report on the importance of a specific factor that is essential for obtaining a successful surfactant-enhanced biodegradation.

Materials and methods

Chemicals and solutions

Radiochemicals used were [3-^{14}C]fluoranthene (Sigma), specific activity 45 mCi mmol^{-1}, >95% radiochemical purity and [U-^{14}C]D-glucose (Sigma), specific activity 6 mCi mmol^{-1}, ≥ 98% radiochemical purity. Nitrilotriacetic acid (NTA) was of 99.5% purity (Sigma). Aqueous stock solution (160 mM; 100 g l^{-1}) of the non-ionic surfactant Triton X-100 (Sigma) was prepared in the respective mineral salts media (see below), solutions were sterilized by autoclaving. The average molar weight of Triton X-100 is 625. The critical micelle concentration (CMC) of Triton X-100 was determined to 0.267 mM (0.166 g l^{-1}) (P.A. Willumsen, unpubl.).

Media

Two different mineral salts media, MS and Bushnell-Haas (BH), were used throughout this study. MS medium contained per liter: 1 g $Na_2HPO_4{\cdot}H_2O$; 3.244 g KH_2PO_4; 2 g NH_4Cl; 0.123 g NTA; 0.2 g $MgSO_4{\cdot}7H_2O$; 12 mg $FeSO_4{\cdot}7H_2O$; 3 mg $ZnSO_4{\cdot}7H_2O$; 3 mg $MnSO_4{\cdot}H_2O$. The final pH was adjusted to 7.2. BH medium (Difco) contained per liter: 1 g $Na_2HPO_4{\cdot}H_2O$; 0.20 g $MgO_4{\cdot}H_2O$; 0.02 g $CaCl_2$; 1 g $(NH_4)_2PO_4$; 1 g KNO_3; 0.05 g $FeCl_3$. Final pH 7.2. Inoculum was prepared in glucose enriched LB medium (LBg) (per liter): 10 g tryptone; 5 g yeast extract; 5 g NaCl and 10 g glucose (pH 7.2). For serial dilution of bacterial cultures, 0.02 M phosphate buffer (3.48 g l^{-1} K_2HPO_4; pH 6.9) was used.

In the mineralization experiments fluoranthene was supplied in excess of its aqueous solubility (0.26 mg l^{-1}, Mackay & Shiu 1977). Fluoranthene-containing medium was prepared by adding acetone-dissolved fluoranthene to sterile flasks at 20 mg l^{-1} calculated medium concentration. The solvent was allowed to evaporate before sterile MS or BH was added. To increase the surface area of non-solubilized PAH-crystals the flasks were subsequently sonicated for 5 min at 140 W in a water bath (Metason 200 (5210E-MT), Struers, Denmark). Surfactants were added from autoclaved stock solutions. To allow fluoranthene saturation of the medium (e.g. 0.26 mg l^{-1} in the absence and 4.50 mg l^{-1} in the presence of 0.48 mM (0.3 g l^{-1}) Triton X-100), flasks were shaken for 24 h before inoculation. Glucose-enriched (100 mg l^{-1}) MS or BH medium was similarly equilibrated 24 h prior inoculation.

Bacterial strain

Sphingomonas paucimobilis, strain EPA505 (Mueller et al. 1990) was used in the glucose and fluoranthene mineralization experiments. The strain was maintained at −80 °C in glycerol (20% vol/vol). To prepare inoculum for batch cultures, cells were pregrown in LBg medium at 25 °C. Growth was determined by optical density at 420 nm. At late exponential growth phase, the cultures were centrifuged (12400 × g, 10 °C, 6 min), resuspended in mineral salts medium and stored at 4 °C for a maximum of 30 min prior use. The number of culturable cells was determined by plating of serial dilutions on LGg plates. Plates were incubated at 25 °C for 3–7 days before enumeration of colony-forming units (CFU).

Mineralization and survival assays

Glucose and fluoranthene mineralization assays were performed in triplicate sterile 250-ml Erlenmeyer

flasks containing 50 ml MS or BH medium with and without Triton X-100 (0.48 mM (0.3 g l^{-1}) unless stated otherwise). 100 mg l^{-1} glucose (specific activity of 1120 Bq mg^{-1} glucose) or 20 mg l^{-1} fluoranthene (specific radioactivity of 1130 Bq mg^{-1} fluoranthene) was also added. After inoculation (initial cell density between 10^5 to 10^9 CFU ml^{-1}), the flasks were sealed with sterile silicone stoppers and incubated at 24 °C on a horizontal shaker at 200 rpm. Sterile uninoculated and autoclaved controls were included. The $^{14}CO_2$ produced through mineralization of glucose or fluoranthene was trapped in 400 μl of 1 M KOH, contained in glass vials suspended from the silicone stoppers by a steel hook. Traps were exchanged at hourly to daily intervals depending on activity. The amount of $^{14}CO_2$ collected was quantified by mixing of the KOH with 1.5 ml Ready Gel Scintillation cocktail (Beckman) and 1.0 ml H_2O. After storage in the dark for at least 4 h, to eliminate chemiluminescence, the samples were counted on a model LS 1801 scintillation counter (Beckman). Counts in controls were never above the background level and correction for abiotic degradation was therefore not necessary. Only $^{14}CO_2$-production exceeding the radiochemical impurity of the applied isotopes was considered significant. At the end of each ^{14}C-carbon mineralization experiments, 1 ml subsample cultures were passed through a 0.2 μm nitrocellulose filter (Sartorius). Radioactivity of the filtrate and radioactivity collected on the filter (i.e. cells and fluoranthene crystals) were measured and corrected for total liquid sample volume. By adding the $^{14}CO_2$ collected, the recoveries in the glucose and fluoranthene mineralization assays were calculated at 89 ± 9% and 99 ± 7%, respectively.

In all experiments, the survival of cells (after 24 and 48 h) was investigated by plating on LBg plates. Numbers of CFUs were measured after 4 days of incubation at 25 °C.

To investigate the effect on fluoranthene mineralization and cell survival of the chelating agent NTA on strain EPA505, BH medium containing fluoranthene was amended with 0.123 g l^{-1} NTA. Potential magnesium limitation was tested by increasing the magnesium concentration in fluoranthene-amended MS medium three fold (from 1.66 to 4.98 mM) by adding autoclaved $MgSO_4$ to autoclaved MS just prior to inoculation. The effect of Ca^{2+}-ions on mineralization of fluoranthene and glucose, and on cell survival was tested by amending the MS medium with increasing amounts of calcium (0–4.1 mM final concentration). To achieve this, autoclaved $CaCl_2$ was added to the autoclaved MS medium containing Triton X-100, just prior to inoculation. Maximum fluoranthene and glucose mineralization rates (calculated as the highest $^{14}CO_2$-production rate) were determined using the statistical program SAS (PROC GLM). This program was also used to evaluate the statistical difference between treatments.

Electron microscopy

Sphingomonas paucimobilis, strain EPA505 was grown in combinations of mineral salts medium (MS and BH), Triton X-100 (0.48 mM; 0.3 g l^{-1}) and calcium (4.1 mM). After five hours of incubation, bacterial samples were prepared for electron microscopy, essentially as described in Glauert 1980. All steps were performed at room temperature. The bacterial cells were prefixed for 1.5 h by addition of 2% (vol/vol) glutaraldehyde directly to the liquid cultures followed by two washings in 0.1 M sodium phosphate buffer (pH 7.0). The washed cells were subsequently fixed over night in 1% osmiumtetraoxide, then chemically dehydrated in 2,2-dimethoxypropane (2 × 10 min) and embedded in Spurrs resin (TAAB, UK). Thin sections (50–80 nm) were cut and stained with lead citrate and uranyl acetate. The bacterial cells on the grids were examined using a JEOL model 100SX transmission electron microscope at an operating voltage of 80 kV.

Results

The growth medium had a strong effect on the tolerance of *S. paucimobilis* EPA505 towards Triton X-100 (Figure 1). When grown in MS medium without Triton X-100, fluoranthene was mineralized at a linear rate (0.26 mg l^{-1} h^{-1}) for the first 24 h of incubation and the number of CFU's remained stable (Figure 1A and C). Addition of Triton X-100, on the other hand, almost completely inhibited the mineralization and resulted in a rapid decline in cell density (Figure 1A and C). At concentrations ≥1.6 mM (1 g l^{-1}) Triton X-100, for instance, the number of CFU's decreased 9 orders of magnitude within 24 h (Figure 1C). Similar to MS medium without Triton X-100, incubation in BH resulted in a linear mineralization rate of fluoranthene (0.41 mg l^{-1} h^{-1}) and in a constant cell number (Figure 1B and D). Addition of Triton X-100, however, increased the maximum fluoranthene mineralization rate to up to 1.2 mg l^{-1} h^{-1} (Figure 1B),

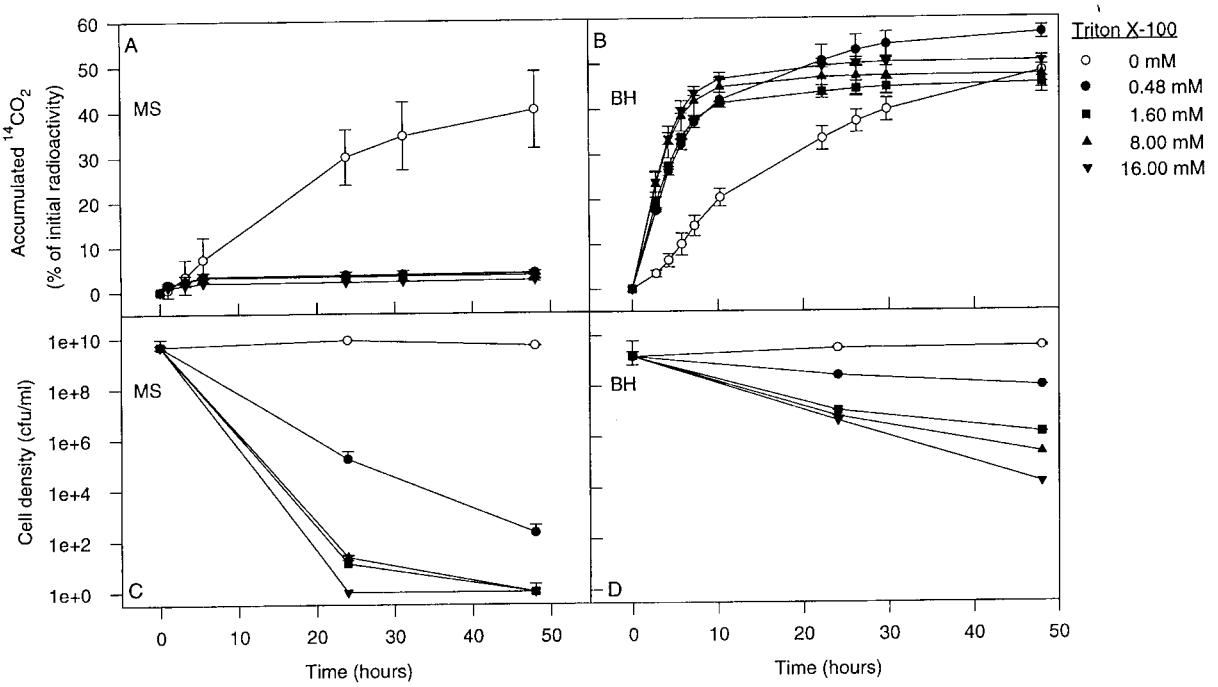

Figure 1. Response of the fluoranthene-degrading strain EPA505 to varying concentrations of Triton X-100 as dependent on medium composition. Fluoranthene mineralization (initial concentration 20 mg l^{-1}) and survival of the cells in the mineral salts media, MS (A and C, respectively) and in BH (B and D, respectively). The initial cell density was 2×10^9 cfu ml^{-1}. Symbols represent treatments, mean ± SD, n = 3.

while the negative effect on cell survival was relatively small compared to in MS (Figure 1C and D). At concentrations ≥ 1.6 mM (1 g l^{-1}) of Triton X-100, cell survival decreased only 2 orders of magnitude in 24 h (Figure 1D). Since the mineralization rate of fluoranthene at 0.48 mM (0.3 g l^{-1}) Triton X-100 in both media was comparable to rates at higher Triton X-100 concentrations, and since cell survival was less affected at this concentration, all further experiments were conducted at 0.48 mM (0.3 g l^{-1}) Triton X-100.

The effect of growth medium on the tolerance of *S. paucimobilis* to Triton X-100 was further verified in glucose mineralization experiments (Figure 2). In the absence of Triton X-100, no statistical difference between maximum mineralization rates ($P < 0.05$) and cell culturability ($P < 0.10$) in MS and BH were found (Figure 2A and B). In BH medium, the bacterial cells were stressed by Triton X-100, although not as strongly as in the MS medium, and the glucose mineralization and in particular survival of strain EPA505 was clearly dependent on the type of mineral salts medium used (Figure 2A and B).

Addition of Triton X-100 accelerated the mineralization of fluoranthene in BH medium independently of the presence of the chelating agent NTA (data not shown). Furthermore, NTA did not affect the survival of the cells in the BH-medium (data not shown).

The presence of Mg^{2+}-ions (1.66 to 4.98 mM) partially reduced the toxic effect of Triton X-100 to strain EPA505 in the MS medium (Figure 3A). The maximum fluoranthene mineralization rates in the presence of Triton X-100 did not differ statistically ($P < 0.05$) from those without Triton X-100 when additional Mg^{2+}-ions were added (Figure 3A). The total amount of fluoranthene that was mineralised during the 48 hours incubation increased from 20 to 37%, as a result of the Mg^{2+}amendment. The culturability of the cells after 48 h was also enhanced by the Mg^{2+}-amendment, although it did not reach the culturability in the cultures without Triton X-100 (Figure 3B).

Calcium significantly alleviated the toxic effect of Triton X-100 to strain EPA505 in MS medium. As expected (see also Figure 1), amendment with Triton X-100 strongly inhibited fluoranthene mineralization in MS without calcium, but the inhibition was reduced significantly by 0.17 mM calcium (Figure 4A). Increasing the calcium concentration up to 4.1 mM final concentration completely removed the inhibition by

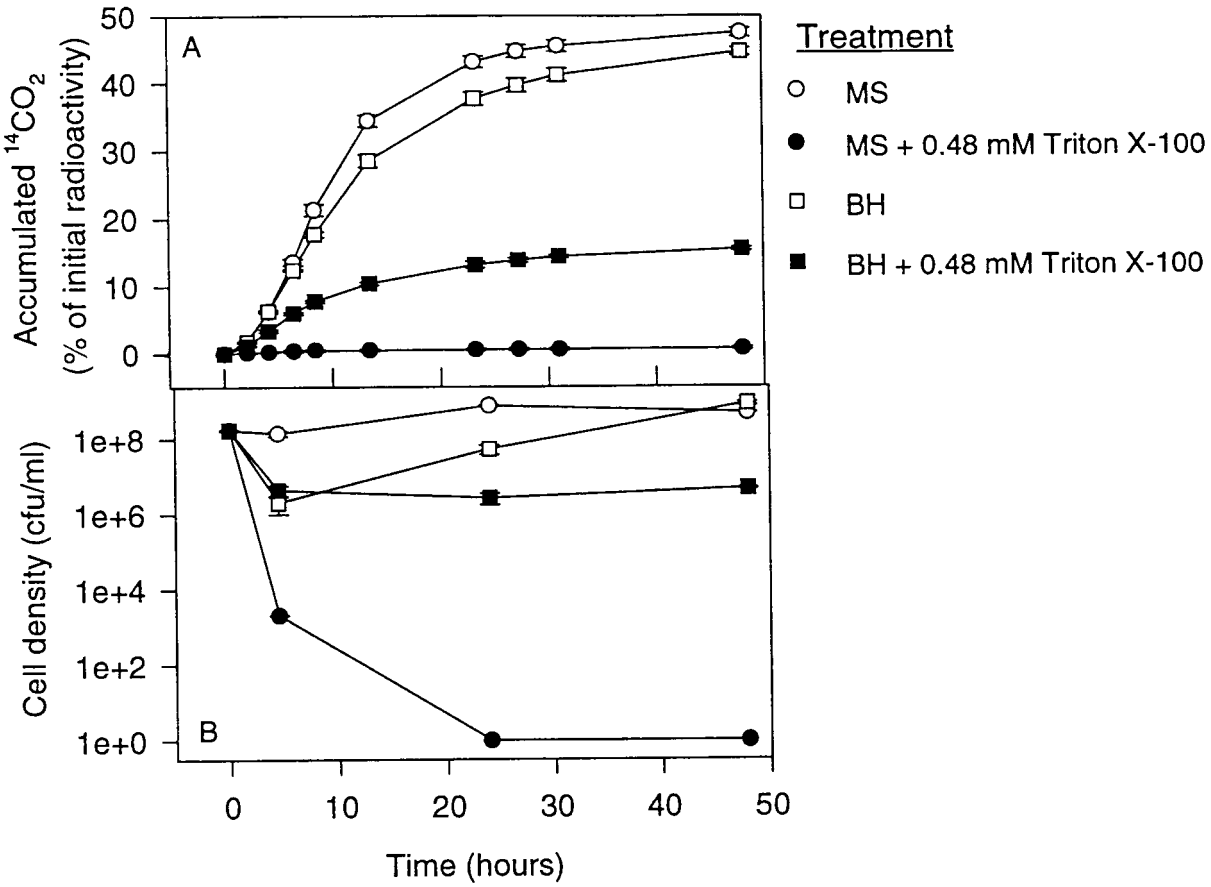

Figure 2. Effect of 0.48 mM (0.3 g l^{-1}) Triton X-100 in the mineral salts media MS and BH on (A) glucose mineralization (initial concentration 100 mg l^{-1}) by strain EPA505 and on (B) survival of the cells. The initial cell density was 1.7×10^8 cfu ml^{-1}. Symbols represent treatments, mean ± SD, $n = 3$.

Triton X-100, resulting in the same Triton-enhanced mineralization pattern as observed in BH medium (Figure 4A). The survival of the cells was similarly improved by the presence of increasing calcium concentrations (Figure 4B). The results obtained in the BH medium (0.18 mM calcium) and in the modified MS-medium (0.17 mM calcium) were, however, significantly different. Rate and extent of fluoranthene mineralization and survival of the cells comparable to those obtained in BH amended with 0.48 mM (0.3 g l^{-1}) Triton X-100 were only achieved in MS amended with 4.1 mM calcium and 0.48 mM (0.3 g l^{-1}) Triton X-100. These results suggest that not all added calcium in the MS medium was directly available to the bacteria probably because of a calcium-phosphate precipitation in the medium, as indicated by the formation of a white precipitate after calcium addition.

Glucose mineralization by strain EPA505 in MS medium with 0.48 mM (0.3 g l^{-1}) Triton X-100 was partly improved by addition of calcium (Figure 5A), whereas the culturability of the EPA505 cells was improved strongly (Figure 5B).

In either mineral salts medium with fluoranthene and Triton X-100, the colour of the growth medium changed from red to reddish brown within two hours after inoculation. When mineralization and cell survival were negatively affected by the presence of the surfactant, this colour change was permanent, whereas the red colour disappeared with the onset of carbon dioxide production.

Transmission electron micrographs, obtained from thin sections of strain EPA505 cells, showed that Triton X-100 significantly affected the density and the distribution of the cytoplasm material. In the absence of Triton X-100, cells cultured in MS (Figure 6A) or

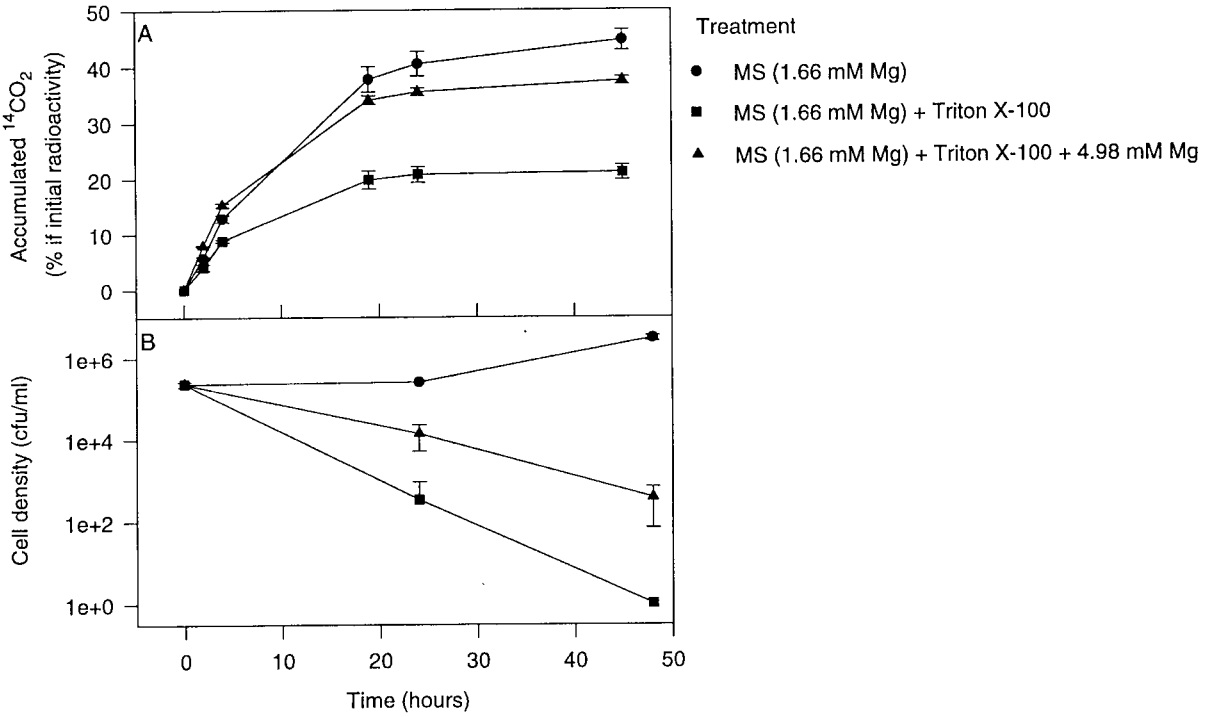

Figure 3. Effect of magnesium addition to MS amended with 0.48 mM (0.3 g l^{-1}) Triton X-100 on (A) fluoranthene mineralization (initial concentration 20 mg l^{-1}) by strain EPA505 and on (B) cellular survival. The initial cell density was 1.2×10^5 cfu ml^{-1}. Symbols represent treatments, mean ± SD, $n = 3$.

in BH (data not shown) had a high electron density in the close vicinity of the cell envelope. Elsewhere within the cell, the cytoplasmic material was evenly distributed (Figure 6A). In the presence of Triton X-100, the electron density of the cytoplasm adjacent to the cell envelope was reduced and the distribution of the cytoplasm was less even, often creating a lumpy appearance (Figure 6B). Strikingly, long voids extending on the inside of the murein layer were visible, indicating an inflated periplasmic space and lack of contact between cytoplamic membrane and cell envelope (Figure 6B). In addition, the outlines of some cells were caved in, suggesting a lack of turgor. However, no visual rupture of cell membranes due to the action of Triton X-100 was observed. Addition of calcium (4.1 mM) partly recovered the distribution of cytoplasm material, even though some crowded (dark) and less dense (white) areas were still visible (Figure 6C). In addition, the periplasmic spaces and the curvature of the cell envelopes appeared to be normal. Likewise, cells grown in BH amended with Triton X-100 (0.48 mM; 0.3 g l^{-1}) had a somewhat uneven appearance (Figure 6D), but to a lesser extent than the cells exposed to the combination of MS and Triton X-100 (Figure 6B). The cytoplasm content of the BH and Triton X-100 exposed cells (Figure 6D) looked more like the cells in MS amended with Triton X-100 and calcium (Figure 6C). Unnormalities in periplasmic spaces and cell envelope curvature were also not observed (Figure 6D).

Discussion

The effect of the non-ionic surfactant Triton X-100 on the culturability of and fluoranthene mineralization by *Sphingomonas paucimobilis* strain EPA505 was investigated. The data showed that Ca^{2+}-ions strongly and Mg^{2+}-ions to a lesser extent reduced the toxicity of Triton X-100 and enhanced the fluoranthene degradation rate.

Except for calcium, MS contained all the ingredients of BH in equal or higher concentrations, plus several additional ions (NH_4^+, Zn^{2+}, Mn^{2+}). Use of minimal amounts of non-toxic chelating agents such as nitrilotriacetic acid (NTA) or ethylene diamine tetra acetic acid (EDTA) are commonly used to prevent the precipitation of metal-ions containing insoluble hy-

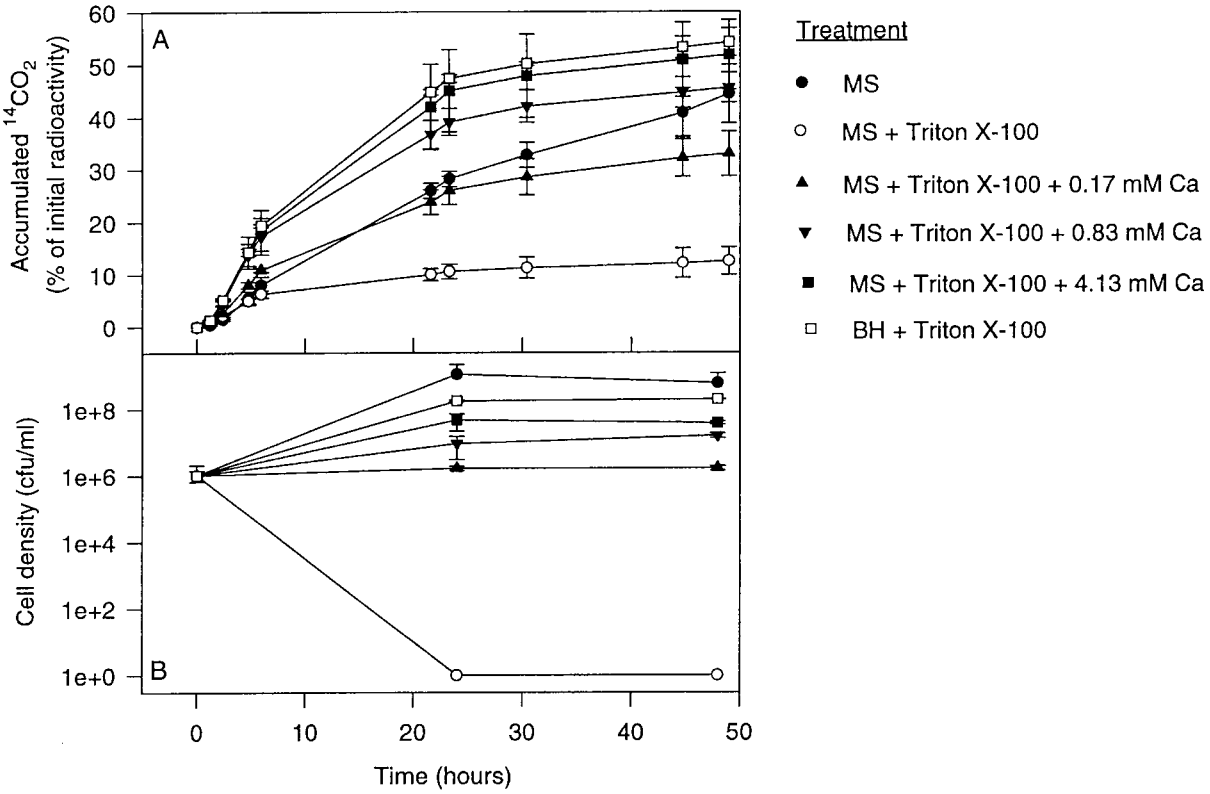

Figure 4. Effect of calcium addition to MS amended with 0.48 mM (0.3 g l^{-1}) Triton X-100 on (A) fluoranthene mineralization (initial concentration 20 mg l^{-1}) by strain EPA505 and on (B) survival of cells. The initial cell density was 2×10^6 cfu ml^{-1}. Symbols represent treatments, mean ± SD, $n = 3$.

droxides or phosphates. However, an over-chelation can result in a metal-limited condition (Cote & Gherna 1994). Only the MS medium contained NTA and the inhibitory effect of Triton X-100 observed in MS medium could thus have been related to micronutrient or magnesium deficiency caused by a chelation effect of NTA and/or to the lack of calcium in the medium. Such nutrient deficiencies not existing in the BH medium. However, the applied concentration of NTA (0.123 g l^{-1}) in the MS-medium was within the recommended range of chelating agents in mineral salts media (Cote & Gherna 1994), suggesting that over-chelation of micro-nutrients or of Mg^{2+}-ions was not a problem. Addition of Triton X-100 accelerated the mineralization of fluoranthene in BH medium independently of the presence of the chelating agent NTA (data not shown). NTA did furthermore not affect the survival of the cells in the BH-medium (data not shown). This ruled out NTA as an influencing factor in Triton X-100 sensitivity.

The divalent ion balance, especially of calcium, is important for several membrane-related processes. The activation and stabilization of a number of extracellular enzymes by 0.1–1.0 mM Ca^{2+} and the influence of calcium in modulation of certain periplasmic and cytoplasm enzymes is well known (Helenius & Simons 1975, Reddy *et al.* 1982). Furthermore, Ca^{2+}-ions have been reported to stabilize the outer membrane of *E. coli* making the cell wall less susceptible to mechanical damage or disaggregation of the lipopolysaccharides and protein components by surfactants (DePamphilis & Adler 1971, Schnaitman 1971a and b). Based on the results presented above, we propose that the difference observed in Triton X-100 toxicity in MS and BH medium was due to the calcium present in the BH medium.

Some non-ionic surfactants, e.g. the Triton X-series, can solubilize lipid bilayers by incorporation into the cell membrane, where they in low concentrations act as “wedges” that destroy the natural orientation of the lipid bilayer resulting in alterations of the

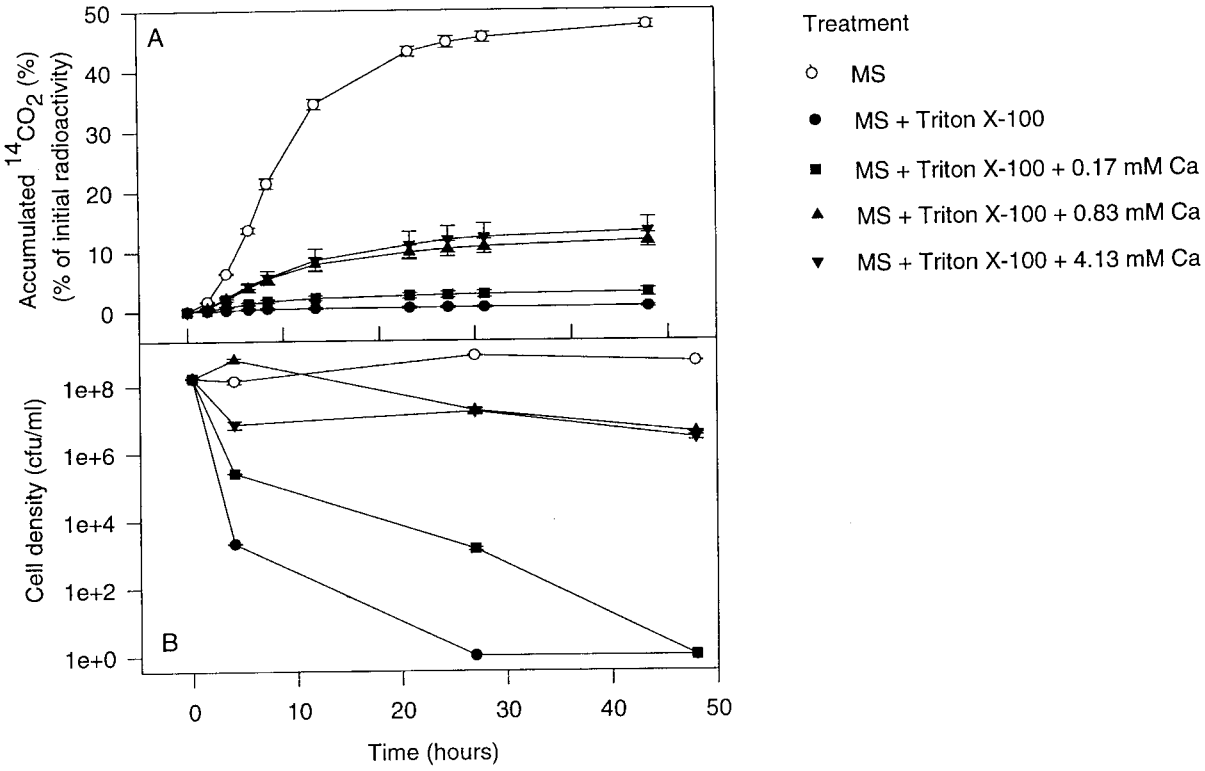

Figure 5. Effect of varying calcium concentrations on (A) glucose mineralization (initial concentration 100 mg l^{-1}) by strain EPA505 and (B) survival of the cells in the presence of 0.48 mM (0.3 g l^{-1}) Triton X-100. The initial cell density was 1.7×10^8 cfu $ml^{-}1$. Symbols represent treatments, mean ± SD, $n = 3$.

membrane permeability. At high surfactant concentrations the membrane becomes saturated with surfactant, causing dissolution of the membrane (Helenius & Simons 1975). The cell membrane permeabilizing capability of Triton X-100 was illustrated by rapid release of red to brownish product(s) into the medium following inoculation of strain EPA505 on fluoranthene-amended medium in the presence of the surfactant. Preliminary tests with strain EPA505 suggest that the red-brownish colour is indeed related to the accumulation of a fluoranthene degradation product resulting from the opening of an aromatic ring at the 9,10 position of fluoranthene (H.P. Pritchard, unpubl.). In BH-experiments with vigorous CO_2 production the red colour was only transient and the disappearance related to the onset of mineralization, whereas in MS-experiments, where both mineralization and survival were adversely affected by the surfactant, the red colour accumulated permanently.

The electron micrographs clearly show that Triton X-100 alters the density and distribution of the cytoplasm in the close vicinity of the cell envelope. In particular, the functioning of the inner membrane appeared to be affected by Triton X-100 under calcium deficiency, as indicated by the visible lack of turgor (Figure 6B). However, no visual damage by Triton X-100 to the outer membrane of strain EPA505 was observed. Schnaitman (1971a and b) found that the resistance of *E. coli* to dissolution by Triton X-100 was partly due to the action of divalent cations that stabilized the structure of the outer membrane by maintaining the impermeability of the cell wall to large molecules. The present work suggests that despite a different chemical composition of the outer membrane (glycosphingolipids instead of lipopolysaccharides (Kawasaki et al. 1994)), *Sphingomonas paucimobilis* strain EPA505 shares the Triton X-100 sensitivity, and the Ca-dependent alleviation thereof, with *E. coli*. Our electron micrographs indicate that in the absence of calcium the adverse effect of Triton X-100 on *Sphingomonas paucimobilis* strain EPA505 extends to the functioning of the cytoplasmic membrane. In two Gram-positive *Mycobacterium* strains,

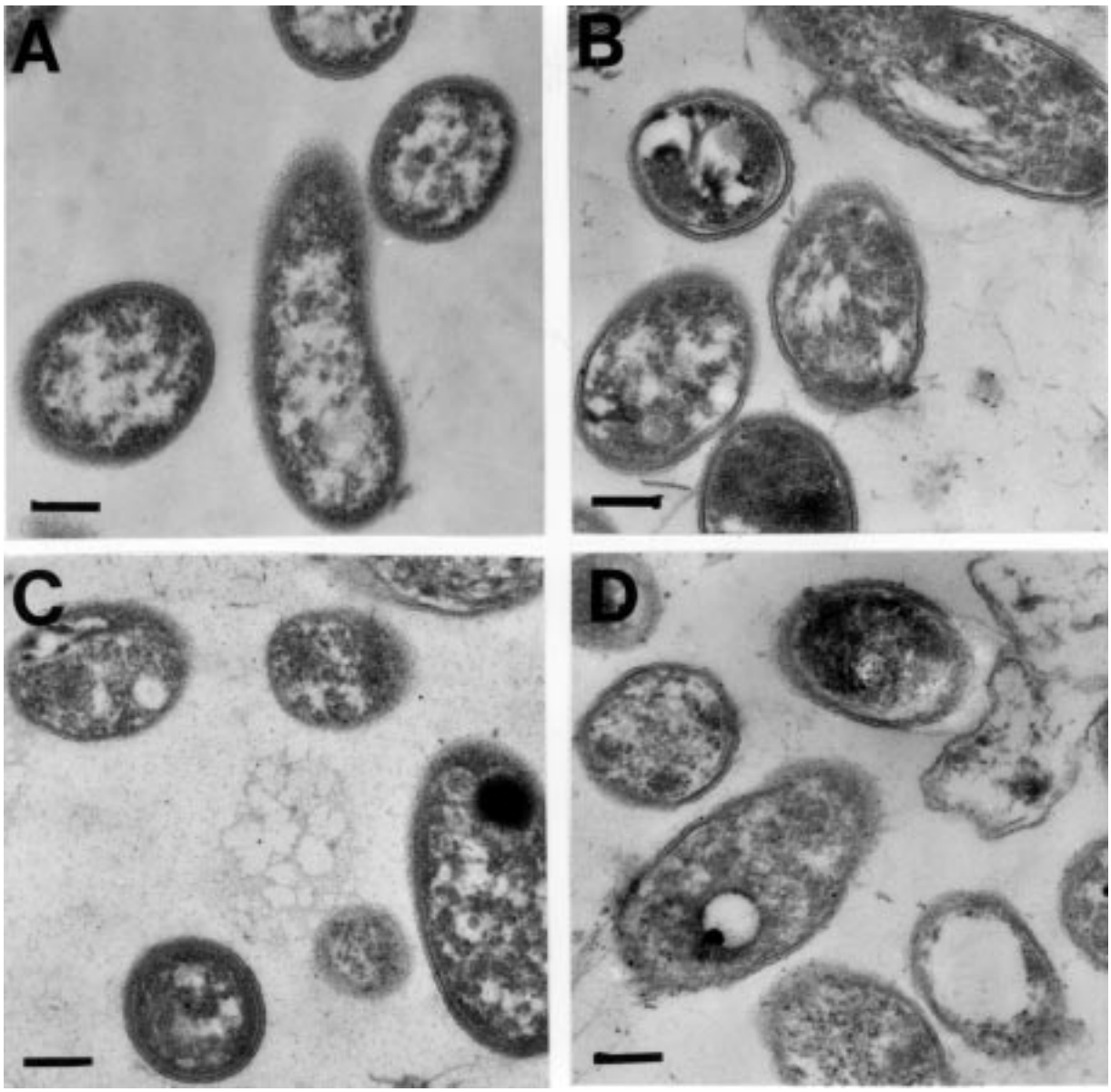

Figure 6. Thin sections of strain EPA505. (A) MS; (B) MS amended with 0.48 mM (0.3 g l^{-1}) Triton X-100; (C) MS amended with 0.48 mM (0.3 g l^{-1}) Triton, and 4.1 mM calcium; (D) BH amended with 0.48 mM (0.3 g l^{-1}) Triton. Bars = 0.025 μm.

Triton X-100 toxicity was not observed, even in the absence of Ca^{2+}-ions (Willumsen et al. 1998).

Bacterial glucose metabolism relies on enzymes in the periplasmic space, some of which are anchored in the cytoplasmic membrane (Schlegel 1993). The location of fluoranthene-degrading enzymes has not been elucidated. However, since glucose metabolism was reduced by Triton X-100 even in the presence of calcium (Figure 2 and Figure 5), the corresponding enzymes appear to be more sensitive against, or less protected from, the surfactant, than the fluoranthene-degrading enzymes. As evidenced by the appearance of red metabolites from fluoranthene in the culture supernatant of all Triton X-100 treatments, irrespective of the calcium concentration, the first fluoranthene-degrading enzyme functions in spite of Triton X-100 toxicity. However, complete fluoranthene mineralization required membrane repair by calcium. This may indicate that the fluoranthene metabolite is produced in the periplasmic space, but further metabolism in-

volves transport through the cytoplasmic membrane. This would also explain the higher sensitivity of glucose mineralization to Triton X-100 toxicity.

In soil bioremediation, surfactants may be added to soil in order to stimulate bacterial degradation of hydrophobic compounds. However, surfactants may in some cases reduce or inhibit biodegradation because of toxicity to the bacteria. Mechanisms suggested to account for the partial or complete inhibition of PAH-mineralization in the presence of surfactants include decreased bioavailability of micelle-solubilized substrate (Laha & Luthy 1991; Volkering et al. 1995; Guha & Jaffé 1996), disruption of cell membranes (Laha & Luthy 1991; Thibault et al. 1996), surfactant interaction with enzymes or proteins essential to the proper functioning of the cells (Laha & Luthy 1991; Volkering et al. 1995) and competitive substrate utilization (Laha & Luthy 1991; Thiem 1994). Our electron micrographs indicate that the effect of Triton X-100 on Gram-negative cells extends to the functioning of the cytoplasmic membrane. Our results are the first report suggesting that divalent ion deficiency in culture medium, Ca^{2+}-ions in particular, and Mg^{2+}-ions to a lesser extent, can account for or aggravate inhibition of PAH-mineralization in surfactant-amended media.

In the study of Laha & Luthy (1991), Thiem (1994), Rock & Alexander (1995) and Thibault et al. (1996) surfactant-related inhibition of PAH-mineralization was observed despite the presence of 0.1 to 0.9 mM calcium in the experimental systems. We found that the inhibitory effect of Triton X-100 on fluoranthene mineralization was completely removed at significantly higher concentrations of calcium (4.1 mM in the MS medium). Based on our results it is likely that the inhibitory surfactant-bacterial cell interactions reported in the above studies was due to insufficient calcium concentrations in the biodegradation systems.

Neither fluoranthene nor glucose mineralization in MS medium was inhibited by the non-ionic surfactant Tween 80 (Willumsen et al. 1998). This suggests that the combination of Triton X-100 and MS medium in particular is unfavourable for the cellular integrity of strain EPA505. The choice of surfactant may thus determine potential inhibitory and/or toxic effects to the bacterial cell.

In conclusion, our results show that if surfactants are used in an attempt to enhance biodegradation by Gram-negative bacteria, amendment with divalent cations, especially Ca^{2+}, may be necessary for successful surfactant-enhanced bioremediation.

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