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Biofilm formation and partial biodegradation of polystyrene by the actinomycete *Rhodococcus ruber*

Biodegradation of polystyrene

Roi Mor · Alex Sivan

Abstract Polystyrene, which is one of the most utilized thermoplastics, is highly durable and is considered to be non-biodegradable. Hence, polystyrene waste accumulates in the environment posing an increasing ecological threat. In a previous study we have isolated a biofilm-producing strain (C208) of the actinomycete *Rhodococcus ruber* that degraded polyethylene films. Formation of biofilm, by C208, improved the biodegradation of polyethylene. Consequently, the present study aimed at monitoring the kinetics of biofilm formation by C208 on polystyrene, determining the physiological activity of the biofilm and analyzing its capacity to degrade polystyrene. Quantification of the biofilm biomass was performed using a modified crystal violet (CV) staining or by monitoring the protein content in the biofilm. When cultured on polystyrene flakes, most of the bacterial cells adhered to the polystyrene surface within few hours, forming a biofilm. The growth of the on polystyrene showed a pattern similar to that of a planktonic culture. Furthermore, the respiration rate, of the biofilm, exhibited a pattern similar to that of the biofilm growth. In contrast, the respiration activity of the planktonic population showed a constant decline with time. Addition of mineral oil (0.005% w/v), but not non-ionic surfactants, increased the biofilm biomass. Extended incubation of the biofilm for up to 8 weeks resulted in a small reduction in the polystyrene weight (0.8% of gravimetric weight loss). This study demonstrates the high affinity of C208 to polystyrene which lead to biofilm formation and, presumably, induced partial biodegradation.

Keywords Biofilm · Polystyrene · *Rhodococcus ruber*

Introduction

Non-biodegradable plastic waste from a wide variety of applications continues to accumulate in the environment, causing a severe pollution problem (Pinto et al. 1998). In this regard, a particularly troublesome synthetic polymer is polystyrene, which is considered to be the most durable thermoplastic polymer. Due to its versatile properties, including resistance to biodegradation, stiffness or flexibility (as required), light weight, good insulation properties, and facile synthesis, polystyrene is used in a wide range of products (Robertson 1993). Sales of polystyrene in the US alone reach 25–30 million tons/year. The major habitats polluted by plastic wastes, including polystyrene waste, are pelagic zones and sedimentary habitats, where most of the plastic debris accumulates in microscopic—but not degraded—fragments. Sampling in different marine habitats has revealed that this plastic debris is ingested by planktonic populations (Thompson et al. 2004), and

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as a consequence, non-biodegradable plastics may accumulate in the food chain, posing an ongoing hazard to wildlife and man alike. To the best of our knowledge, to date, there are no reports in the literature on the effective biodegradation of pure polystyrene. One of the early attempts to biodegrade polystyrene was that of Kaplan et al. (1979), who showed biodegradation of as little as 0.5% (measured as evolved $^{14}CO_2$) after 11 weeks incubation of ^{14}C-labeled polystyrene with mixed populations of bacteria, fungi, and invertebrates. In another study, Otake et al. (1995) found no signs of degradation of a polystyrene sheet buried in soil for 32 years. There has been some success in improving the biodegradability of polystyrene by preconditioning the polymer by inserting a degradable molecule into the polystyrene hydrocarbon backbone. Incubation of a polystyrene–starch copolymer with *Bacillus coagulans* (Kiatkamjornwong et al. 1999) and of polystyrene–lignin copolymers with fungi (Milstein et al. 1992) showed that the polymers supplemented with starch or lignin were indeed degraded. A similar approach was tested by Galgali et al. (2002), who reported that insertion of various mono- or disaccharides into the carbon backbone of polystyrene increased its biodegradability. We have taken a different approach: since it is well known that biofilm is the preferred mode of living of most microbial terrestrial and aquatic populations (Costerton et al. 1995; Soren Molen), we set out to exploit this characteristic for the degradation of plastics. We recently isolated a strain (C208) of the actinomycete *Rhodococcus ruber* that was found to be capable of colonizing polyethylene and utilizing it as sole source of carbon and energy (Gilan (Orr) et al. 2004; Sivan et al. 2006). In the present study we have monitored the kinetics of biofilm formation by C208 on polystyrene, determined the physiological activity of the biofilm and tested its capacity to degrade pure polystyrene.

Materials and methods

Polystyrene

Three forms of polystyrene were used in this study: (a) pure standard polystyrene flakes comprising a mixture of polymers of two different molecular weights, 4,000 and 200,000 (Sigma Cat. Number 33,165-1); (b) polystyrene powder produced by grinding the above polystyrene flakes in a coffee grinder and screening to obtain 10–50 μM particles; and (c) ELISA 96-well microtiter plates manufactured from pure polystyrene (C96 MicroWell[TM], Nalgene Nunc International, USA). The flakes were used in assays for the biodegradation of polystyrene and for the determination of respiratory activity and protein content of the biofilm. The microtiter plates were used in experiments aimed at comparison of the effects of different factors, such as temperature, pH, carbon and nitrogen sources, or additives, such as non-ionic surfactants on biofilm formation.

Bacterial culture and growth conditions

The polyethylene-degrading bacterium *R. ruber* strain C208, previously isolated in our laboratory (Gilan (Orr) et al. 2004), was used in this study. Cultures of C208 (50 ml) were maintained at 35°C in nutrient broth (NB; Difco, USA), either in 250 ml flasks incubated on a rotary shaker (120 rpm) or on nutrient agar (NA) plates. Cultures used for quantification of the biofilm were incubated in the 96-well polystyrene plates. Unless otherwise specified, all assays for evaluating the bacterial biofilms were performed in a synthetic medium (SM), contained the following elements (g in 1 l of distilled water): NH_4NO_3 1.0; K_2HPO_4 1.0; $MgSO_4 \cdot 7H_2O$ 0.2; KCl 0.15; $CaCl_2 \cdot 2H_2O$ 0.1; and each of the following microelements: 1.0 mg l^{-1} $FeSO_4 \cdot 6H_2O$, 1.0 mg l^{-1} $ZnSO_4 \cdot 7H_2O$, and 1.0 mg l^{-1} M_nSO_4.

Polystyrene biodegradation assay

Biodegradation tests were performed on samples of pure polystyrene flakes that had been dried overnight at 60°C, weighed, disinfested (30 min in 70% ethanol) and added to flasks (about 1.0 g of polystyrene per flask) each containing 50 ml of SM. Flasks were inoculated with 2 ml of a mid-exponential-phase culture of *R. ruber* (C208) maintained in Nutrient Broth (NB; Difco) and washed in SM to remove medium and cellular soluble debris. Cell density of the inoculum was adjusted to 1.5×10^8 colony-forming units (CFU) ml. Flasks containing non-inoculated SM medium supplemented with polystyrene served as the control.

   To facilitate accurate measurement of the weight of the residual polystyrene the bacterial film colonizing the polystyrene surface was removed by supplementing the cultures with 2% (v/v) aqueous sodium

dodecyl sulfate (SDS) solution and incubating the flasks for 4 h at 50°C. This detergent was found to be efficient for removal of the biofilm (Sivan et al. 2006). The polystyrene samples were collected on a filter paper, rinsed with distilled water and then dried overnight, at 60°C, before they were finally weighed. The initial weights of the pre-incubated polystyrene samples were measured following the same procedure mentioned above.

Estimating bacterial biomass of the biofilm

Biofilm formation by C208 was analyzed in polystyrene 96-well microtiter plates containing 200 µl of SM per well. The polystyrene plate thus served as the sole carbon source. Each well was inoculated with C208 to a final density of 10^6 cells ml^{-1}, and the plates were incubated at 35°C for up to 1 week. After incubation, the medium was discarded, and the biofilm that had formed on the surface of the well was washed to remove medium remnants and planktonic cells. Since the bacterial cells were strongly attached to the polystyrene surface, it was impossible to estimate the population density by standard techniques, such as direct cell counting or plating. Therefore, the population density of the biofilm on the polystyrene surface was estimated by the crystal violet (CV) staining method of O'Toole and Kolter (1998). This method is based on the direct correlation between bacterial biomass and absorbance (at 600 nm) of ethanol extract of the cells. Each well was supplemented with 0.2% of a CV solution in 95% ethanol and the plates were kept for 15 min at room temperature. The staining solution was discarded, and the plates were washed with distilled water to remove the excess stain. Finally, 95% ethanol was added to the well to extract the stain from the bacteria adhering to the well surface. The extraction procedure was repeated until no detectable CV was obtained (at 600 nm). The extracts were combined and the total CV was determined. We have modified the original method by transforming the concentrations of CV to CFU by using a standard curve of preset cell densitiy values versus measured OD (600 nm) data of CV as follows: aliquots of C208 suspension, with increasing cell concentrations, were stained with a 0.2% solution of CV in 95% ethanol according to the staining procedure described above. A linear standard curve of cell density (CFU) versus

absorbance (600 nm) was obtained for cell extracts from the same samples and used to quantify the biofilm cell density of C208. However, since CV staining does not distinguish between viable and dead cells, the data were proportionally normalized to the values of living cells obtained after staining with the LIVE/DEAD® *Bac*Light™ (Bacterial Viability Kit Probes, Eugen, Oregon, USA), according to the manufacturer's instructions. Estimation of the live/dead cell-ratio was performed with an epifluorescent microscope with a fluorescein isothiocyanate (FITC) filter set.

Estimation of the protein content of biofilms

The total protein content of the biofilm biomass was determined after alkaline hydrolysis, as follows. Polystyrene flakes were sampled from flasks containing SM cultures of C208, washed with water to remove medium debris and boiled for 20 min in 4.0 ml of 0.5 N NaOH. The extracts were centrifuged to precipitate fragments of cell debris and the protein content in the extract was determined spectrophotometrically at 280 nm.

Biofilm respiration measurements

The metabolic activity of the biofilm was measured with the fluorescent redox probe 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), which facilitates direct monitoring of actively respiring bacteria. The colorless non-fluorescent CTC is readily reduced by the bacterial electron transport system (ETS) to fluorescent insoluble CTC-formazan (CTCF). The respiration was monitored by measuring the level of fluorescence. Bacteria were cultured in flasks containing 50 ml of SM, as described above, amended with 15 polystyrene flakes, each weighing about 200 mg. At daily intervals, a polystyrene flake was transferred from the bacterial culture to a 96-well microtiter plate containing 200 µl of SM and 5.0 mM of CTC. The plates were incubated for 4 h at 28°C before measuring the fluorescence of CTCF (excited at 450 nm and visualized at 630 nm in a Polar Star-Galaxy fluorimeter).

Scanning electron microscopy (SEM) for biofilm analysis

Polystyrene samples were suspended in cultures of C208 and removed at prescheduled times to observe

the bacterial colonization of the polystyrene. The samples were washed free of excess medium for 20 min in 0.1 M phosphate buffer (pH 7.2), fixed in 2% glutaraldehyde for 2 h, washed twice (30 min each) in 50% ethanol, incubated overnight in 70% ethanol, and finally washed again (30 min × 3) in 100% ethanol. After fixation, the samples were vacuum dried, coated with gold, and scanned in a Quanta 20 SEM.

Statistics

All experiments were repeated three times. The data shown are of one representative experiment. Experiments performed in the 96-well plates were set in eight replicates (wells). All other experiments consisted of three replicates. In all experiments the calculated standard deviation of the mean are shown. The biodegradation experiments were analyzed by analysis of variance ($P = 0.05$).

Results

Biofilm formation on polystyrene by strain C208 of *R. ruber*

Incubation of *R. ruber* in SM containing polystyrene flakes as the sole source of organic carbon resulted in the adhesion of cells to the polystyrene surface and the formation of biofilm (data not shown). Colonization and kinetics of biofilm formation were tested in in polystyrene microtiter plates. These 96-well plates enabled accurate monitoring of adhesion and proliferation of multiple biofilms (Fig. 1). Biofilm formation showed a pattern similar to that of growth curves obtained from liquid cultures of planktonic cells, demonstrating exponential and stationary phases of growth and a generation time of about 1 day (Fig. 1). Maximal cell density in biofilms, produced either on the polystyrene microtiter plates or on polystyrene flakes, was obtained after about 4 days of incubation. A representative SEM photomicrograph of a four-day-old biofilm is shown in Fig. 2.

The 96-well microplate culture method was used for determining the optimal temperature and the nitrogen source for sessile and planktonic cultures. These tests showed no differences in the optimal
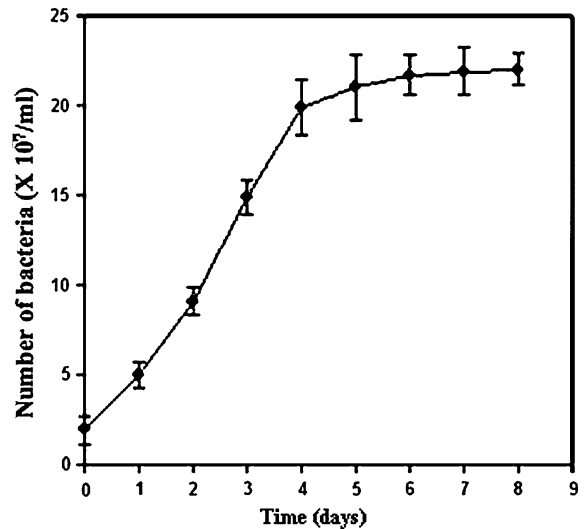

Fig. 1 Growth of biofilm (◆) of *R. ruber* in 96-well microtiter polystyrene plates in a mineral medium deficient in organic carbon (200 μl/well). The biofilm on the well surface was quantified by CV staining of the bacterial biomass. A calibration curve was used to convert optical density (600 nm) values of CV stained samples to CFUs of viable bacteria. Data represent means of eight replicates ±SD
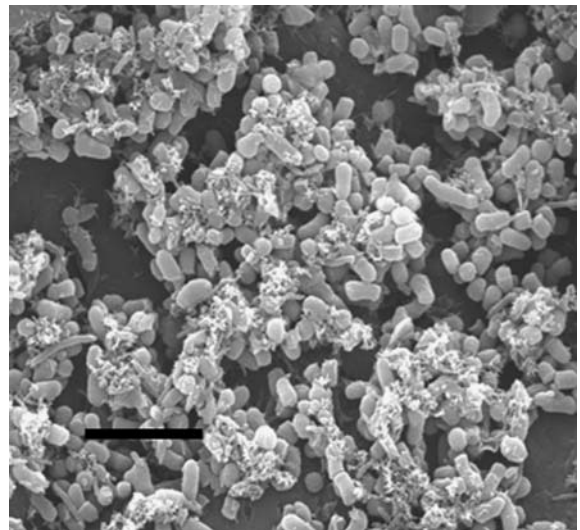

Fig. 2 Scanning electron microscope (SEM) photomicrograph of a four-day-old biofilm of *R. ruber* on polystyrene flakes. Bar = 5 μm

temperature (35°C), nitrogen source (KNO_3), and pH (7.0) between standard planktonic culture and of sessile biofilm cultures (data not shown).

To determine whether an additional carbon source would have an impact on growth and on biofilm formation, we screened 19 carbon compounds for their ability to support growth of C208. Only the addition of mannitol or glucose (0.5 gl^{-1}; w/v) enhanced biofilm formation by 42.2 and 51.1%, respectively, while supplementation with ethanol or sucrose had no effect on biofilm growth (Fig. 3).

Biofilm is the preferred mode of growth of *R. ruber* C208

Growth kinetics of biofilm formation on polystyrene flakes was monitored by quantifying the total proteins extracted from the biofilm. The data in Fig. 4 showed a biphasic pattern of biofilm formation on the polystyrene flakes. The first phase was characterized by a steep increase in protein content during the first 4 days of incubation, reflecting an increase in the biofilm biomass. This phase was followed by a second period in which the protein content remained constant. In contrast, the planktonic cells did not exhibit a biphasic growth pattern: the protein content in cell extracts showed a rapid linear decrease
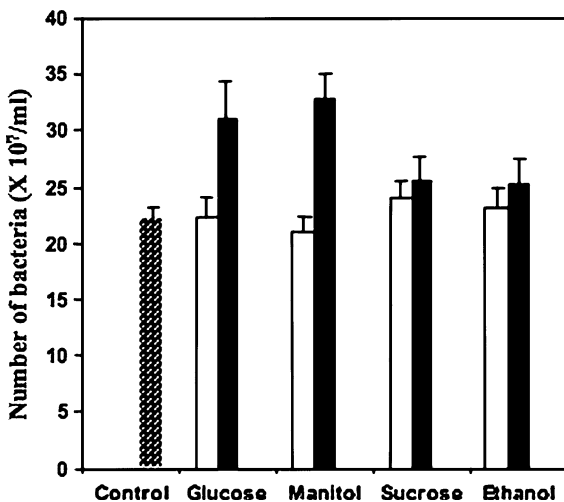
Fig. 4 Biofilm (○) and planktonic (■) protein contents of *ruber* cultured in a mineral medium supplemented with polystyrene flakes (2%;w/v) as the sole organic carbon in the medium. Polystyrene flakes incubated in a sterile medium served as controls. Data represent the means of three replicates ±SD

(Fig. 4), which indicates the strong affinity of C208 to the polystyrene.

Viability and physiological activity of the biofilm

The reduction of CTC to CTC-formazan, driven by the ETS, was used to determined the respiration level of the biofilm. Respiration of the biofilm on the polystyrene flakes increased sharply during the first 3 days of incubation, followed by a constant respiration rate throughout the experiment (Fig. 5a). On the other hand, the planktonic cells showed a reduction in respiration activity with time, starting on the 4th day of incubation (Fig. 5a). Furthermore, the reduction in CFU serves as additional evidence for the loss of cell viability of the planktonic cells (Fig. 5b).

Effect of mineral oil and non-ionic surfactants on biofilm formation

Since polystyrene is hydrophobic and strain C208 has been characterized as a partially hydrophobic bacterium (Gilan (Orr) et al. 2004), we attempted to study the effect of mineral oil and non-ionic surfactants on biofilm formation on polystyrene. Three non-ionic surfactants that did not affect growth of C208 (Tween 20, Tween 60, and Tween 80) were evaluated as potential enhancers of biofilm formation (data not shown). When cultures of C208 supplemented with

Fig. 3 Effect of polystyrene supplementation with additional organic compounds, at concentrations of 0.2 (□) and 0.5 g l^{-1} (■), on *R. ruber* biofilm formation after nine days of growth in 96-well microtiter polystyrene plates (200 μl/well) in a mineral medium deficient in organic carbon. The biofilm on the well surface was quantified by CV staining of the bacterial biomass. A calibration curve was used to convert optical density (600 nm) values of CV-stained samples to CFUs of viable bacteria. Data represent the means of eight replicates ±SD
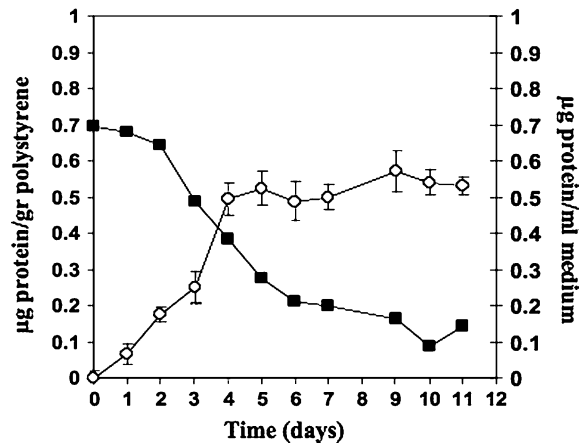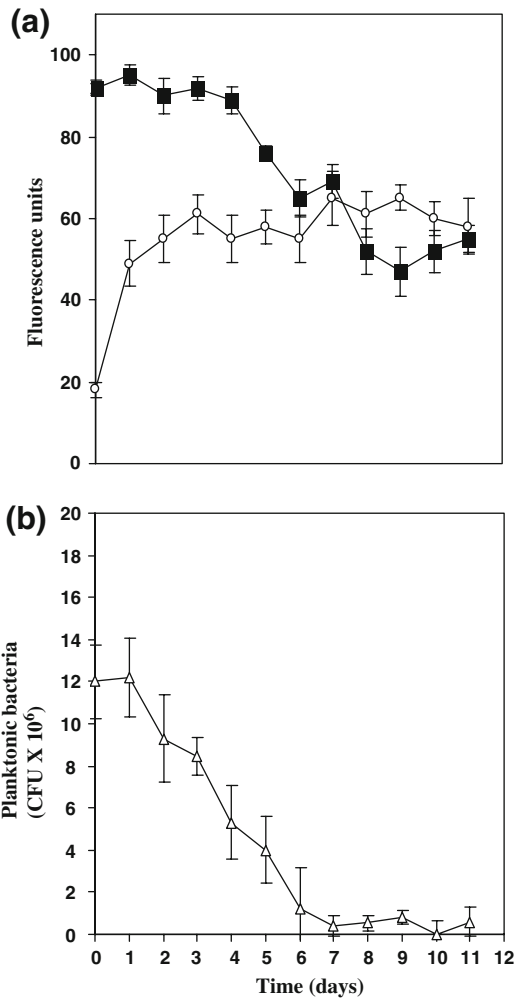
Fig. 5 Respiration of biofilm (○) and planktonic (■) cells of *R. ruber* cultured in a eight mineral medium containing 1% (w/v) of polystyrene flakes (**a**). Respiration was measured in terms of the intracellular reduction of cyano-2,3-ditolyl tetrazolium chloride (CTC) by cytochromes of the ETS to the fluorescent compound CTC-formazan, thereby facilitating direct monitoring of respiration. Viability of the planktoic population expressed as CFU (△; **b**). Data represent the means of three replicates ±SD

Fig. 6 Effect of the surfactant Tween 80 (■) and mineral oil (●) on *R. ruber* (C208) biofilm formation. Aliquots (200 μl) of mineral medium (SM) containing 0.005% of Tween 80 or mineral oil were poured into 96-well microtiter polystyrene plates. Non amended SM served as control (○) Quantification of biofilm on the well surface was estimated by CV staining of bacterial biomass. Optical density (600 nm) values of CV-stained samples were transformed and expressed as CFUs. Data represent means of eight replicates ±SD

Biodegradation of polystyrene by C208

The first indication of the ability of *R. ruber* C208 to cause biodegradation of polystyrene stems from its capacity to proliferate and form colonies on SM agar plates containing pure polystyrene powder as the sole organic carbon in the medium compared with polystyrene-free control plates containing the same SM (Fig. 7). In an attempt to quantify the degradation C208 was cultured with polystyrene flakes 4 and 8 weeks; this resulted in reduction of 0.5% and 0.8%, respectively, in polystyrene gravimetric weight (Fig. 8).

Discussion

This study shows the colonization, biofilm formation and, presumably, partial biodegradation of polystyrene by *R. ruber* (C208). According to the consensus model, formation of microbial biofilms is usually initiated when a planktonic culture proliferates until it reaches a cell density that triggers—via quorum sensing signals—attachment of cells to a surface,

0.005% of Tween 20 were cultured in polystyrene microtiter plates, it was evident that the surfactant had no effect on the growth pattern and total biofilm produced (versus non-supplemented controls) during the first 24 h of incubation. However, addition of the same concentration of mineral oil led to increase of about 130% in the total biofilm bacterial count (Fig. 6). Similar results were obtained when cultures of C208 containing Tween 20 and mineral oil were incubated for 30 days (data not shown).
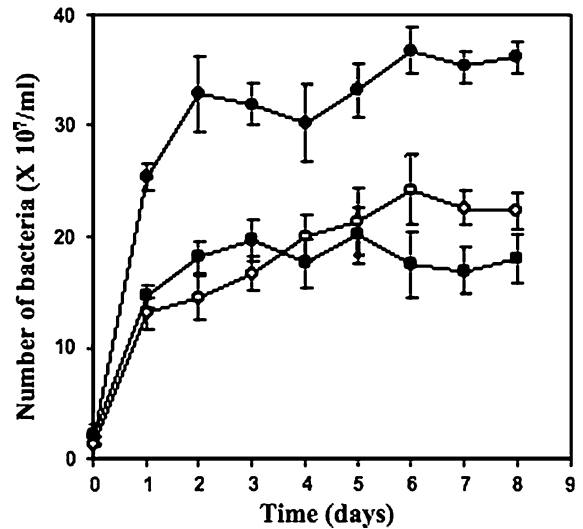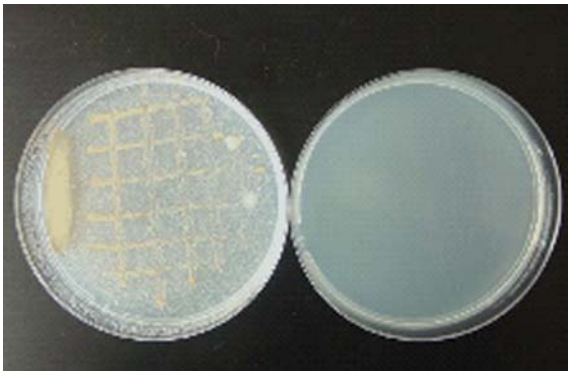
Fig. 7 Four-day-old *R. ruber* culture in a Petri dish on mineral medium containing 1.0% of polystyrene powder as the sole source of organic carbon in the medium
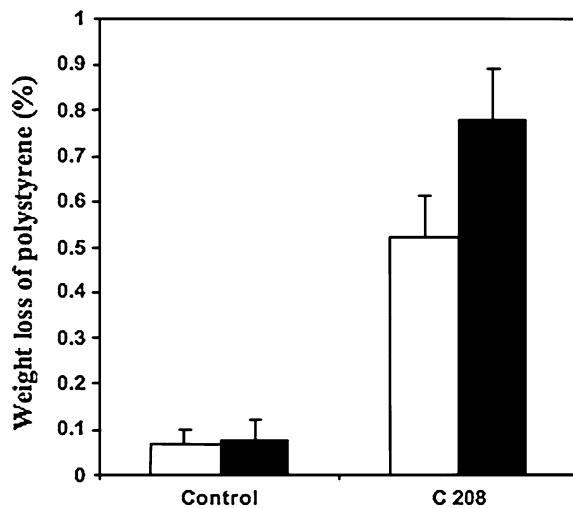

Fig. 8 Gravimetric weight loss of polystyrene following4 (□) and 8 weeks (■) incubation with *R. ruber* in a mineral medium (50 ml) containing pre-weighed polystyrene flakes (3–5 g per flask) as the sole source of organic carbon in the medium. Data represent means of three replicates ±SD

thereby initiating the formation of microcolonies that will eventually develop the 3D sessile structure of the mature biofilm (Costerton et al. 1995). Therefore, this process requires that the planktonic culture be grown in a rich medium to facilitate the quorum sensing signaling needed for induction of cell attachment to the substrate (Tolker-Nielsen et al. 2000). However, this scenario is not necessarily common to all biofilm-producing bacteria. In contrast to the consensus model, the polystyrene in our study served not only as a substrate for the formation of biofilms but also as a carbon source for C208. Thus,

employing carbon starvation by using a medium in which the polystyrene was the sole source of organic carbon apparently resulted in cell death, as could be seen in the simultaneous decrease in biomass, respiration and CFU in the planktonic cells, which reflects the absence of an available carbon source in the medium. In contrast, prolonged incubation (of up to 8 weeks) of C208 with polystyrene resulted in a dense biofilm on the polystyrene surface which may have led to a partial degradation (about 0.8% weight loss) of the polymer. Alternatively, the limited growth together with the low degradation rate could have resulted from consumption of low molecular impurities of the polymer surface. Similarly, the capacity of *R. ruber* C208, to form and maintain a stable and biologically active biofilm, for 11 days, on polystyrene may also be due to utilization of low molecular substances in the polymer. These findings suggest that the formation of biofilms on hydrophobic polymers, such as polystyrene, may be promoted by carbon starvation. Indeed, there is evidence in the literature demonstrating a correlation between carbon starvation and bacterial hydrophobicity (Sakharovski et al. 1999). Similarly, Sanin et al. (2003) reported changes in bacterial surface hydrophobicity in response to carbon starvation. It was shown that with carbon starved culture bacterial isolates including *R. corallinus* became more hydrophobic and more adhesive than with non-starved cells. These findings may explain the high affinity of C208 cells for the polystyrene and raise the possibility that the low carbon availability in C208 cultures may also enhance hydrophobic interactions and biofilm development. Similarly, in a recent study, Sivan et al. (2006) showed that the biofilm of *R. ruber* formed on polyethylene showed high viability and even after 60 days of incubation adhered to the polyethylene without any supplement of external carbon.

One of the drawbacks in microbial biofilm research is the lack of direct and reliable methods for quantifying the biomass of the biofilm. Such techniques are useful for various physiological and biochemical studies. Here, we present a simple procedure for determining the total biofilm biomass and the growth kinetics. This method is based on the biofilm staining technique of O'Toole and Kolter (1998) but is modified to express the optical density readings of CV to CFU of living cells, thus facilitating a good estimation of the number of living cells in

the biofilm at particular time points. Furthermore, the use of pure polystyrene microtiter plates that served as the substrate for biofilm formation (the biofilm staining method of inner surface of each well) enabled in situ measurement of biofilm size, density, and viability. The pattern of biofilm proliferation in the multi-well plate was similar to that of standard growth curves (depicted from bacterial growth in suspended cultures). The increased accuracy of this method may be useful in comparative studies of kinetics of biofilm formation in several bacteria simultaneously.

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