J Ind Microbiol Biotechnol (2007) 34: 5–8
DOI 10.1007/s10295-006-0087-2

ORIGINAL PAPER

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Influence of high salinity on biofilm formation and benzoate assimilation by *Pseudomonas aeruginosa*

Received: 29 July 2005 / Accepted: 19 January 2006 / Published online: 21 February 2006
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Abstract *Pseudomonas* species were used in bioremediation technologies. In situ conditions, such as marine salinity, could limit the degradation of hydrocarbons and aromatic compounds by the bacteria. Biofilm ability to tolerate environmental stress could be used to increase biorestoration. In this report, we used scanning confocal laser microscopy and microtiter dish assay to analyse the impact of hyperosmotic stress on biofilm formation by *Pseudomonas aeruginosa*. We used benzoate as the sole carbon source and the effect of the stress on its degradation was also studied. Hyperosmotic shock inhibited the biofilm development and decreased the degradation of benzoate. The osmoprotectant glycine betaine partially restored both the biofilm formation and benzoate degradation, suggesting that it could be used as a complement in bioremediation processes.

Keywords Osmotic stress · Glycine betaine · Rhamnolipid · Biofilm · Benzoate

Introduction

Pollutions by aromatic compounds unfortunately occur frequently as a consequence of increased industrial activities. Many environmental aromatic pollutants have been reported to be degraded by a variety of microorganisms, which contain various dioxygenases capable of cleaving aromatic rings [7]. However, biodegradation is subject to variations of environmental conditions that make it very slow, especially in extreme environments like sea, where two major limitations are the poor accessibility of bacteria to many hydrocarbons and seawater osmolarity. To overcome the first limitation, some bacteria produce biosurfactants, which are amphiphilic compounds. These biosurfactants reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids and increase the surface areas of insoluble compounds, leading to increased mobility, bioavailability and subsequent biodegradation [6]. The best studied biosurfactants are the rhamnolipids of *Pseudomonas aeruginosa*. They contain one or two 3-hydroxy fatty acids of various lengths, linked to a mono- or di-rhamnose moiety [9]. They are required for the maintenance of the pillar and water channel structures seen in biofilms. However, rhamnolipids inhibit biofilm development and disrupt both cell-to-cell and cell-to-surface interactions [4].

Increased osmolarity is also an important factor that inhibits biodegradation, especially in marine environment [11]. It causes considerable decrease in the cytoplasmic water activity, which results in the inhibition of a variety of physiological processes, ranging from nutrient uptake to DNA replication [3]. Interactions between a cell and a surface can be regulated by increased osmolarity. Biofilm formation by *Escherichia coli* and some other bacterial species is inhibited by high osmolarity of the surrounding medium [15]. However, these conditions activate EnvZ/OmpR, a two-component signalling pathway promoting stable cell–surface interactions [15]. In most natural environments, bacteria–surface association leads to the formation of a biofilm, which is the prevailing microbial lifestyle [17]. The biofilm organization provides bacteria increased resistances to various antimicrobial agents. Furthermore, growth within a biofilm seems to induce a general stress response, which protects the cells from environmental stresses [10]. Microorganisms can cope specifically with hyperosmotic stress by intracellular

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accumulation of compatible solutes [2, 3]. Osmoprotectants are compatible solutes accumulated from the surrounding media which enhance considerably the osmotolerance of microorganisms. Glycine betaine (GB) is the most efficient osmoprotectant used by various bacteria [2].

In order to improve the biodegradation of aromatic pollutants under hypersaline environment, we investigated here the effect of hyperosmotic stress and the presence of GB on biofilm formation and on degradation of benzoate, a key metabolite in the pathway of numerous hydrocarbons catabolism by *P. aeruginosa*.

Materials and methods

Bacterial strains and culture conditions

Pseudomonas aeruginosa PAO1 was the wild-type strain used in the present study [8]. Plasmid pSMC21 (GFP^+, Ap^R, Kn^R) constitutively expresses *gfp* and was used to label the strains for microscopy studies [1]. The culture media were LB [13] or the phosphate-limited minimal medium PLM63, with 10 mM benzoate as the sole carbon source (A. Bazire, A. Dheilly, F. Diab, D. Morin, M. Jebbar, D. Haras and A. Dufour, submitted). During precultures of *P. aeruginosa* (pSMC21), kanamycin was used at the concentration of 500 $\mu g\ ml^{-1}$. Cultures were performed at 37 °C with vigorous shaking, except where indicated. Osmotic shock (0.5 M NaCl) was applied by adding one volume of 1 M NaCl-containing medium (preheated at 37 °C) to NaCl-free cultures. When osmotic shock was performed in the presence of the osmoprotectant glycine betaine (GB), 2 mM GB was included in the NaCl-containing medium. The final GB concentration was thus 1 mM. For unstressed controls, one volume of NaCl-free medium was added to the cultures.

Microtiter dish assay of biofilm formation

PLM63 (100 μl per well of a polypropylene microtiter dish) was inoculated (1:50) with an overnight LB preculture of *P. aeruginosa* PAO1, and the dish was incubated at 37 °C without shaking for 24 h. Osmotic shock was then applied as described above, and the dish was further incubated for 24 h. After removing the medium and planktonic cells, the biofilm was macroscopically visualized by the addition of 200 μl of a 0.1% solution of crystal violet to each well, incubation for 10 min at room temperature, washing three times in distilled water, distaining in ethanol and measuring the absorbance at 570 nm [4].

Scanning confocal laser microscopy analyses of biofilms

Pseudomonas aeruginosa PAO1 (pSMC21) biofilms were formed on glass microscope slides, immersed in 30 ml of medium in 9 cm diameter petri dishes. Incubation and osmotic stress were performed as described above. The slides were washed twice in phosphate-buffered saline [13] and biofilms were observed using a Leica DM6000B confocal microscope (Leica Microsystems, Heidelberg, Germany), with the immersion 63X objective. The green fluorescent protein (GFP) was excited with the 488 nm line of an argon laser, and emission was collected at 500–550 nm. Confocal stacks were collected, and images were processed using Leica Confocal Software and Adobe Photoshop.

Catabolism of benzoate

Bacteria were grown in PLM63 medium containing 10 mM of [U-^{14}C]benzoate (Sigma, with a specific activity of 1.22 GBq $mmol^{-1}$). Osmotic shock (0.5 M NaCl) was performed as described above. Bacteria were harvested from 1 ml of culture by centrifugation at room temperature for 10 min at 13,000×*g*, 3.5 and 5.5 h after stress application Biodegradation rate was then determined by measuring the radioactivity remaining in supernatant, the radioactivity in soluble and insoluble cell components and in evolved CO_2. Briefly, the cells pellet was extracted twice with 80% (vol/vol) ethanol at room temperature for 10 min, and the radioactivity was determined as previously described [16] in the ethanol-soluble fraction (ESF) and the ethanol-insoluble fraction (EIF), which contain soluble cytoplasmic molecules and insoluble macromolecules, respectively. For trapping $^{14}CO_2$, 40 μl KOH (6 N) was used. The radioactivity was determined using a Packard Tri-Carb 1600 TR scintillation counter.

Results

Hyperosmotic stress reduces biofilm formation

We previously observed that *P. aeruginosa* PAO1 grown in liquid PLM63 medium with benzoate as carbon source is able to resume its growth after a hyperosmotic stress (0.5 M NaCl) applied during the exponential growth phase (OD_{600} 0.3) (A. Bazire et al., submitted). Here, we examined the effect of osmotic stress on biofilm formation by adding NaCl 24 h after inoculation and incubating for 24 additional hours. Using the microtiter dish assay, we observed that the biofilm amount on polypropylene was reduced by about two-thirds in osmotic stress condition (Fig. 1). We also used confocal laser microscopy to study the architecture of biofilm formed on glass surface. To do so, *P. aeruginosa* PAO1 strain was first transformed with plasmid pSMC21, which constitutively expresses the *gfp* gene [4]. In the absence of stress, the biofilm was sparse with a thickness of about 12 μm (Fig. 2). Hyperosmotic stress resulted in a 50% thickness diminution of the biofilm (Fig. 2B). In

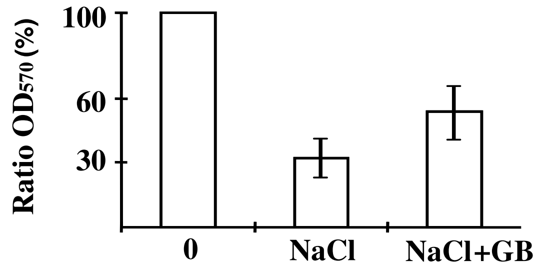

Fig. 1 Effect of hyperosmotic shock (0.5 M NaCl) on the adhesion of *P. aeruginosa*. Bacteria were grown in PLM63 medium in microtiter dish and one volume of medium containing NaCl or no NaCl was added after 24 h of growth. Adhesion was revealed by crystal violet coloration and compared together by measuring OD_{570}. The bacteria were unstressed (0), submitted to osmotic shock (NaCl) or submitted to osmotic shock in the presence of 1 mM glycine betaine (NaCl GB). The graph was representative of two independent experiments

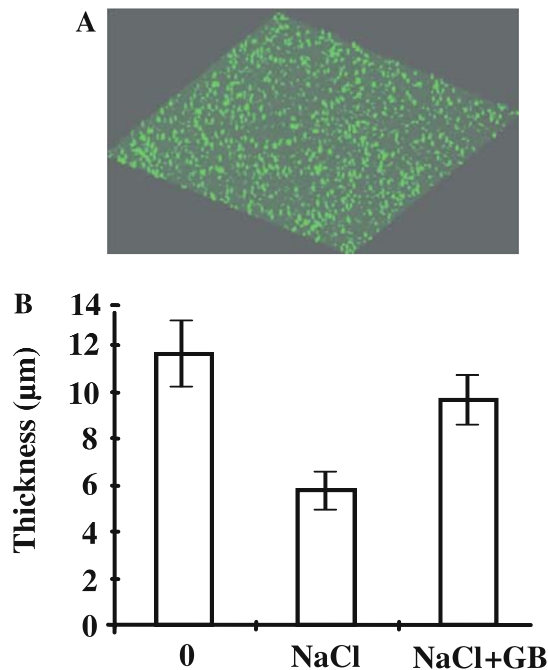

Fig. 2 Effect of hyperosmotic shock on the thickness of *P. aeruginosa* PAO1 biofilms. GFP-labelled strain was grown in a petri dish in PLM63 and adhesion was done on glass. One volume of medium containing NaCl or no NaCl was added after 24 h of growth. The bacteria were unstressed (**a**) (**b**, 0), submitted to osmotic shock (**b**, NaCl) or submitted to osmotic shock in the presence of 1 mM glycine betaine (**b**, NaCl GB). Thickness values are means of data of three images from two independent experiments

both experiments, the presence of the osmoprotectant GB partially restored biofilm formation: on polypropylene microplate walls, the amount of biofilm-forming bacteria was only 1.8-fold lower than in unstressed conditions and 1.5-fold higher than in PLM63 with NaCl but no GB (Fig. 1). Similarly, the biofilm thickness on glass surface in medium containing NaCl and GB was 10 μm, which was intermediate between the thicknesses observed in unstressed condition and in osmotic stress without osmoprotectant (Fig. 2B).

Degradation of benzoate by cell suspensions under salt stress

To know the ability of *P. aeruginosa* to metabolize benzoate under an osmotic stress, bacterial cells were incubated with 10 mM [U-^{14}C]benzoate. Without salt in the medium, about 37 and 50% of [U-^{14}C]benzoate was degraded after 3.5 and 5.5 h of incubation, respectively (Fig. 3). At 5.5 h, 20% of the initial radioactivity was recovered in the form of $^{14}CO_2$, whereas about 50% of the initial radioactivity remained in the medium. Addition of a final concentration of 0.5 M NaCl to the medium provoked a twofold weaker decrease in benzoate from the medium (18 and 27% at 3.5 and 5.5 h of incubation, respectively) (Fig. 3). However, CO_2 production was slightly less than in the control, showing that osmotic shock did not affect benzoate metabolism significantly. This was in agreement with previous results showing that growth rates of stressed culture were close to that of unstressed controls (A. Bazire et al., submitted). GB was used as osmoprotectant to combat the adverse effect caused by elevated external osmotic

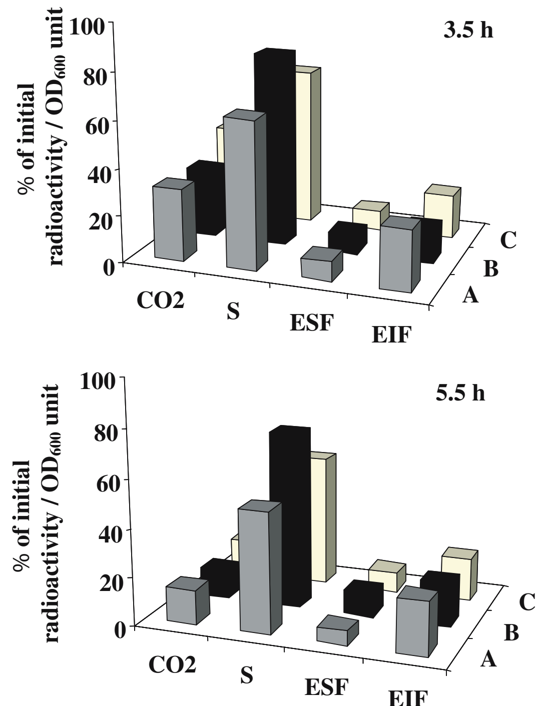

Fig. 3 Mineralization of benzoate by *P. aeruginosa* under hyperosmotic stress. No salt was added to the control (**a**), 0.5 M NaCl was added in the medium to perform salt stress without GB (**b**) and with 1 mM GB (**c**). At the indicated time, cells were harvested and extracted as indicated in the text. Percentages of initial radioactivity were determined in $^{14}CO_2$, supernatant (*S*), ethanolic soluble fraction (*ESF*) and ethanolic insoluble fraction (*EIF*)

pressure. Benzoate degradation was more efficient than in stressed culture without GB, since the degradation rate was 45% at 5.5 h of incubation and $^{14}CO_2$ production was higher than the other conditions (Fig. 3).

Discussion

Biodegradation is an attractive method for remediation of contaminated sites because of its economic viability and environmental soundness. However, despite decades of research, successful bioremediation of aromatic compounds contaminated saline environments remains a challenge. Since cells within biofilm seem to be adapted to environmental stresses [10], we studied here the effect of hyperosmotic shock on biofilm formation by *P. aeruginosa* grown with benzoate as the sole carbon source. In PLM63, biofilm structure was tin and equivalent to that observed in FAB citrate medium by De Kievit et al. [5]. This observation could be explained by the strong similarity of the two media: they contain low phosphate concentrations and other carbon sources than glucose. When osmotic shock was applied, biofilm formation was altered, but not completely inhibited, unlike in other non-halophilic cells such as *E. coli* [15]. We suggest that adhesion, which is the first step of biofilm formation, was completed when osmotic shock was applied, and that biofilm growth still kept place despite the high salinity, although more slowly. It was previously shown that a high rhamnolipid concentration changes the biofilm structure [4] and that hyperosmotic conditions in PLM63 prevented rhamnolipid production (A. Bazire et al., submitted). In our conditions, biofilms were too thin to allow us to observe biofilm architecture variations.

Since rhamnolipids are biosurfactants which increase the assimilation of water-insoluble molecules [6], we observed if mineralization of benzoate was affected by inhibition of rhamnolipid production under osmotic stress. In a medium without salt, *P. aeruginosa* actively degrades benzoate. When bacteria were placed in a medium with higher salt concentration (0.5 M NaCl), mineralization was slightly inhibited. To perform osmotic shock, 0.5 M NaCl was chosen because it is close to the seawater one ($<3\%$). Such salinities do not require typically halotolerant microorganisms [12]. On the other hand, to perform osmotic shock, cells were taken at the exponential phase, so in a favourable physiological state that allows a better resistance to salinity. Otherwise, the lack of rhamnolipid production does not seem to affect benzoate assimilation. In the same conditions, osmoprotectant GB had a light role to protect cells from the deleterious effects of salinity. So it will be interesting to apply a higher osmotic shock to see a more spectacular osmoprotectant effect. Several studies showed that high osmoprotectant concentrations play an important role to protect cells from the inhibitory effect of salt and other denaturing agents [2, 3]. GB protects in vitro several enzymes from denaturation provoked by dehydration or high salt concentrations [11, 14], so this compound should be useful as a complement to microorganisms for bioremediation in seawater.

Acknowledgements This work was supported by the Region Bretagne, FEDER funds, and the Ministère de la Recherche et de la Technologie (RITMER grant and doctoral fellowships to A.B. and F. D.). We thank G. A. O'Toole for the kind gift of plasmid pSMC21. We thank A. Dufour for his critically reading manuscript.

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