Motility and chemotaxis of *Pseudomonas* sp. B4 towards polychlorobiphenyls and chlorobenzoates

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Abstract

The polychlorinated biphenyl (PCB)-degrading *Pseudomonas* sp. B4 was tested for its motility and ability to sense and respond to biphenyl, its chloroderivatives and chlorobenzoates in chemotaxis assays. *Pseudomonas* sp. B4 was attracted to biphenyl, PCBs and benzoate in swarm plate and capillary assays. Chemotaxis towards these compounds correlated with their use as carbon and energy sources. No chemotactic effect was observed in the presence of 2- and 3-chlorobenzoates. Furthermore, a toxic effect was observed when the microorganism was exposed to 3-chlorobenzoate. A nonmotile *Pseudomonas* sp. B4 transformant and *Burkholderia xenovorans* LB400, the laboratory model strain for PCB degradation, were both capable of growing in biphenyl as the sole carbon source, but showed a clear disadvantage to access the pollutants to be degraded, compared with the highly motile *Pseudomonas* sp. B4, stressing the importance of motility and chemotaxis in this environmental biodegradation.

Introduction

Bioremediation of soil contaminated with polychlorinated biphenyls (PCBs) is an attractive clean-up strategy due to its potential to mineralize pollutants and to be inexpensive (Ohtsubo *et al.*, 2004). Many genetic, enzymological, and biochemical analyses of PCB-degradative pathways have provided the basis for the engineering of specific enzymes and have been used to modify genetically microorganisms to improve their performance in bioremediation of PCBs (Tiedje *et al.*, 1993; Timmis *et al.*, 1994; Pieper & Reineke, 2000). *Burkholderia xenovorans* LB400 (formerly known as *Burkholderia fungorum* LB400) is one of the most-studied and effective aerobic PCB degraders known (Denef *et al.*, 2004; Goris *et al.*, 2004) and many other biphenyl-utilizing microorganisms with capacity to degrade PCBs to different extents have been isolated (Bopp, 1986; Bartels *et al.*, 1999). However, little is known about the physiological and ecological adjustments that help PCB-degrading bacteria to degrade contaminated soil-sorbed chemicals (Chávez *et al.*, 2006).

Most motile bacteria can sense and respond to low concentrations of organic compounds in their environment by the process of chemotaxis. In the last few years, there have been several reports on bacterial species responding chemotactically to different environmental pollutants, many of which are considered to be serious ecological problems (Pandey & Jain, 2002; Parales & Harwood, 2002). This phenomenon may be widespread among other chemicals and have important significance for the potential of these microorganisms as biodegraders. There is also evidence that chemotaxis can not only enhance biodegradation but also promotes the formation of microbial consortia (Law & Aitken, 2003; Wu *et al.*, 2003), presumably by rapidly bringing cells into close contact with degradable substrates. Other physiological properties such as high-affinity uptake systems, adhesion to solid surfaces, biosurfactant production and biofilm formation have been suggested to reduce the distances between cells and solid chemicals and thus enhance substrate bioavailability (Wick *et al.*, 2002; Wu *et al.*, 2003; Ohtsubo *et al.*, 2004).

The objective of this study was to determine whether biphenyl (B), chlorobiphenyls (CBs), PCB congeners and some of the intermediates of these biodegradations such as benzoate and its chloroderivatives (CBAs), which are excreted by some PCB-degrading bacteria (Potrawfke *et al.*, 1998), are recognized as chemotactic effectors by *Pseudomonas* sp. B4. The results reported here indicate that biphenyl, CBs, PCBs and benzoate are attractants for *Pseudomonas* sp. B4. However, a nonmotile *Pseudomonas* sp. B4 transformant was not able to access the same compounds in capillary assays. To our knowledge, this is the first report of biphenyl-

utilizing bacterial chemotaxis toward CBs and PCBs. Our findings are of importance as bacterial strains with the ability to sense the presence of PCBs or their metabolic intermediates may have an increased growth and survival advantage that could contribute to the bioremediation of PCB-contaminated environments.

Materials and methods

Chemicals

Biphenyl was purchased from Merck (Hohenbrunn, Germany), benzoic acid and chlorobenzoates were obtained from Sigma (St Louis, MO) and chlorinated biphenyls and PCBs from Accustandard Inc. (New Haven, CT).

Microorganisms and growth conditions

Pseudomonas sp. B4 was isolated from the Elbe River (Germany), contaminated with PCBs. It was initially characterized as a biphenyl-utilizing bacterium belonging to the Gamma Phylogenetic cluster and to the *Pseudomonas* Taxonomic cluster, being closest to *Pseudomonas putida* by a 97.7% 16S rRNA gene sequence similarity (Bartels *et al.*, 1999). *Burkholderia xenovorans* LB400, *Pseudomonas* sp. B4 and its nonmotile polyphosphate-deficient derivative (Chávez *et al.*, 2006) were grown aerobically at 30 °C on Luria–Bertani (LB) rich medium and M9 minimal salts medium (Sambrook *et al.*, 1989; Chávez *et al.*, 2004) supplemented with 0.1% biphenyl, 1% glucose or 0.1% benzoate as the sole carbon sources. The strains were also grown using chlorinated biphenyls and PCBs (at a final 1 mM concentration). Biphenyl and its chloroderivatives are poorly soluble in water and therefore the indicated concentrations in the figures are only nominal, most of the compounds being most likely saturated at concentrations higher than their solubility limits. When these insoluble carbon sources were used, they were dissolved in iso-octane (0.2% final concentration in each test and control assay). The microorganisms did not grow in the presence of iso-octane alone, which was nontoxic and showed no chemotactic properties compared with the compounds tested in the swarming or capillary assays (see below).

Swarming assays

Qualitative measurement of motility was done essentially as described by Harwood *et al.* (1994) using plates consisting

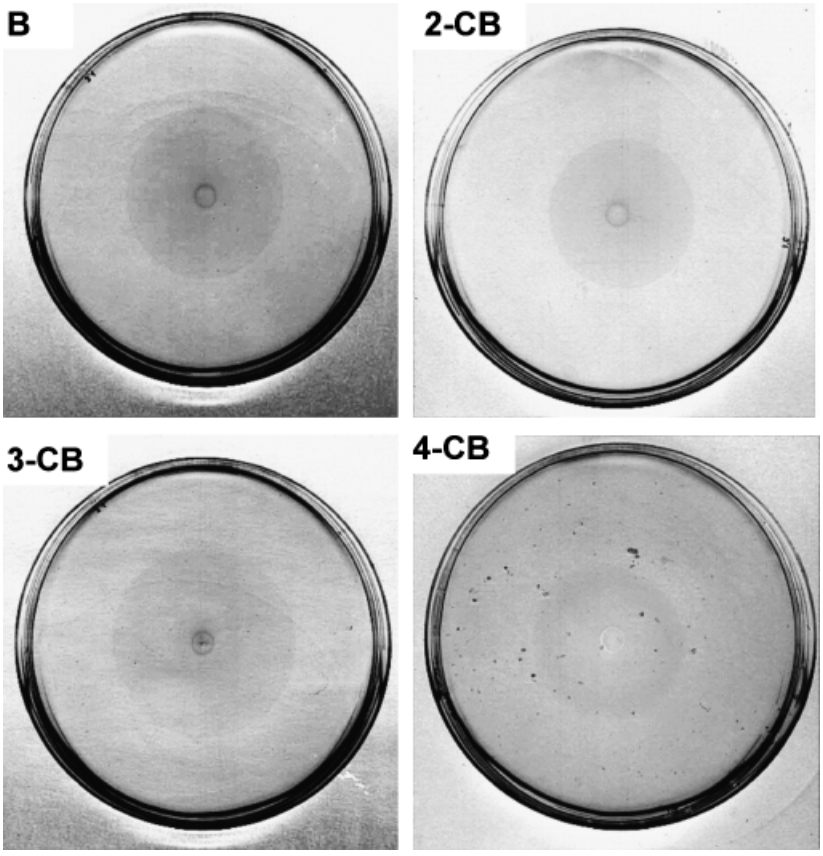

Fig. 1. Chemotactic swarming responses of *Pseudomonas* sp. B4 to different chlorobiphenyls. Cells grown in minimal medium were inoculated in plates containing the same medium supplemented with biphenyl (B) and different CBs (2-CB, 3-CB and 4-CB) as the sole carbon sources by stabbing them at a point corresponding to the centre of the swarm ring and incubated for 72 h at 30 °C. The negative print of the photographs is shown for better contrast of the swarming rings.

of M9 minimal medium containing 0.3% Noble agar (Difco) and the chemoattractant carbon source at a final concentration of 1 mM. To test for toxic effects of some compounds on the bacteria, we used the same swarming plates, combined with a chemical-in-plug system as initially described by Tso & Adler (1974). Plugs of 2% agar that contained the compound to be tested as a toxic (100 mM) were incubated at 30 °C until the swarming rings reached the plugs containing the test compounds (72 h). The inhibitory effect was seen as a clear area around the plug.

Modified capillary chemotaxis assay

The capillary assay with the modifications described previously (Mazumder *et al.*, 1999) was done using a disposable 200-μL pipette tip as a chamber for holding 100 μL of bacterial suspension (usually 1×10^7 cells^{-1}) in chemotaxis buffer (10 mM Tris-HCl, pH 7.4). A 2-cm 25-gauge needle (Becton Dickinson) was used as the chemotaxis capillary and was attached to a 1-mL tuberculin syringe (Becton Dickinson) containing a 200-μL portion of the compound to be tested in chemotaxis buffer in the presence of 0.2% iso-octane or a control containing only the chemotaxis buffer and 0.2% iso-octane. After 90 min incubation at room temperature the needle syringe was removed from the bacterial suspension and the content diluted and plated in LB medium. Accumulation of bacteria in the capillaries was calculated as the average from the CFUs obtained in duplicate plates and the results were expressed as the mean of at least three separate capillary assays for each determination. The relative chemotaxis index (RCI) was calculated as the ratio of the bacteria that entered the test capillary to that in the control capillary. An RCI of 2 or greater has been described as significant with this method (Mazumder *et al.*, 1999).

Results and discussion

Motility assays in swarm plates

The spreading behaviour of the colonies of *Pseudomonas* sp. B4 in solid agar strongly suggested it was highly motile compared with *B. xenovorans* LB400. Although *B. xenovorans* LB400 was originally described as a motile microorganism (Goris *et al.*, 2004), this microorganism was nonmotile as observed by phase contrast light microscopy (not shown). The same behaviour has been reported previously by other researchers (Nielsen *et al.*, 2000). By using the qualitative motility assay in swarming plates, *Pseudomonas* sp. B4 showed a clear motility for casaminoacids compared with the nonmotile *Pseudomonas* sp. B4 (Chávez *et al.*, 2006) or *B. xenovorans* LB400 (not shown).

It has recently been described that chemotaxis of *P. putida* G7 towards naphthalene dissolved in a nonaqueous-phase liquid substantially increases the rates of mass transfer and degradation of these hydrophobic pollutants (Law & Aitken, 2003). To study the importance of motility in accessing some of the pollutants that *Pseudomonas* sp. B4 can transform or use as carbon sources, we started checking the motility of these microorganisms in soft agar swarm plates of M9 medium supplemented with different CBs. A clear swarm response was seen (Fig. 1) in plates containing biphenyl (B), 2-CB, 3-CB or 4-CB. These results were in agreement with the capacity of these cells to grow on these compounds as the sole carbon sources.

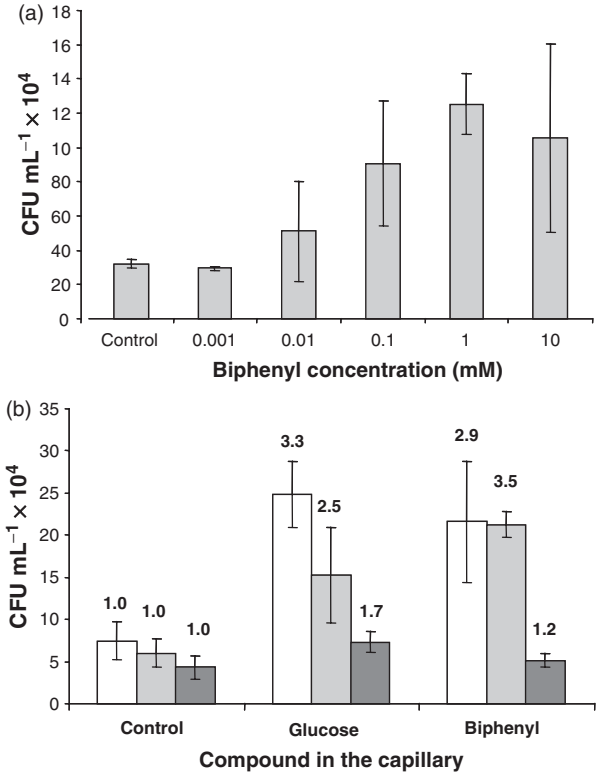

Fig. 2. (a) Chemotactic response of *Pseudomonas* sp. B4 towards increasing biphenyl concentration. Cells grown to the mid-exponential phase in 1% glucose were collected by centrifugation and resuspended in chemotaxis buffer. After centrifugation, the chemotactic response of *Pseudomonas* sp. B4 towards biphenyl was examined. (b) Effect of growth medium in chemotaxis of *Pseudomonas* sp. B4 toward different compounds. Cells were grown to the mid-exponential phase in minimal medium containing 1 mm biphenyl (white bars), 1% glucose (light grey bars) as the sole carbon source or in LB medium (dark grey bars). The capillary assay was done as in (a). The bacterial cells attracted by the indicated compounds were determined after duplicate plating in LB medium. Error bars indicate the SDs based on three different replicated experimental values. Numbers on top of each bar indicate the relative chemotactic indexes.

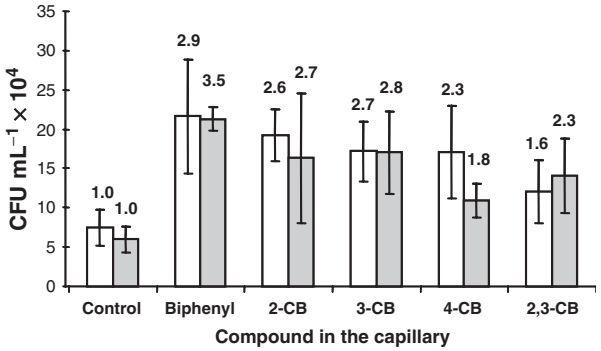

Fig. 3. Chemotaxis of *Pseudomonas* sp. B4 towards several organochlorine compounds. Cells were grown to the mid-exponential phase in minimal medium containing 1 mm biphenyl (white bars) or 1% glucose (grey bars) as the sole carbon sources. For the capillary assay, cells were collected by centrifugation and resuspended in chemotaxis buffer. The bacterial cells attracted by biphenyl, 2-CB, 3-CB, 4-CB and 2, 3-CB were determined after plating the cells in LB medium. Error bars indicate the SDs for three replicated experimental values. Numbers on top of each bar indicate the relative chemotactic indexes.

Quantitative chemotaxis responses of *Pseudomonas* sp. B4 to different compounds

The capillary assays showed that *Pseudomonas* sp. B4 cells accumulated in capillaries containing increasing biphenyl concentrations with a maximum number of cells apparently obtained at 1 mM biphenyl nominal concentration inside the capillary, as higher concentrations gave results with low reproducibility (Fig. 2a). As *Pseudomonas* sp. B4 is able to grow in biphenyl and several CBs (Chávez *et al.*, 2004), it may be possible that previous growth of the microorganism in biphenyl would induce a chemotactic response towards these compounds. Chemotaxis of *Pseudomonas* sp. B4 toward biphenyl or CBs (Figs 2b and 3) was not induced by previous growth of the cells in these substrates compared with that seen by growing previously the cells in glucose and therefore can not be considered an 'inducible type' (Parales & Harwood, 2002). On the other hand, chemotaxis of *Pseudomonas* sp. strain NCIB 9816–4 and *P. putida* G7 towards naphthalene is known to be induced by previous growth in the chemoeffector (Grimm & Harwood, 1997).

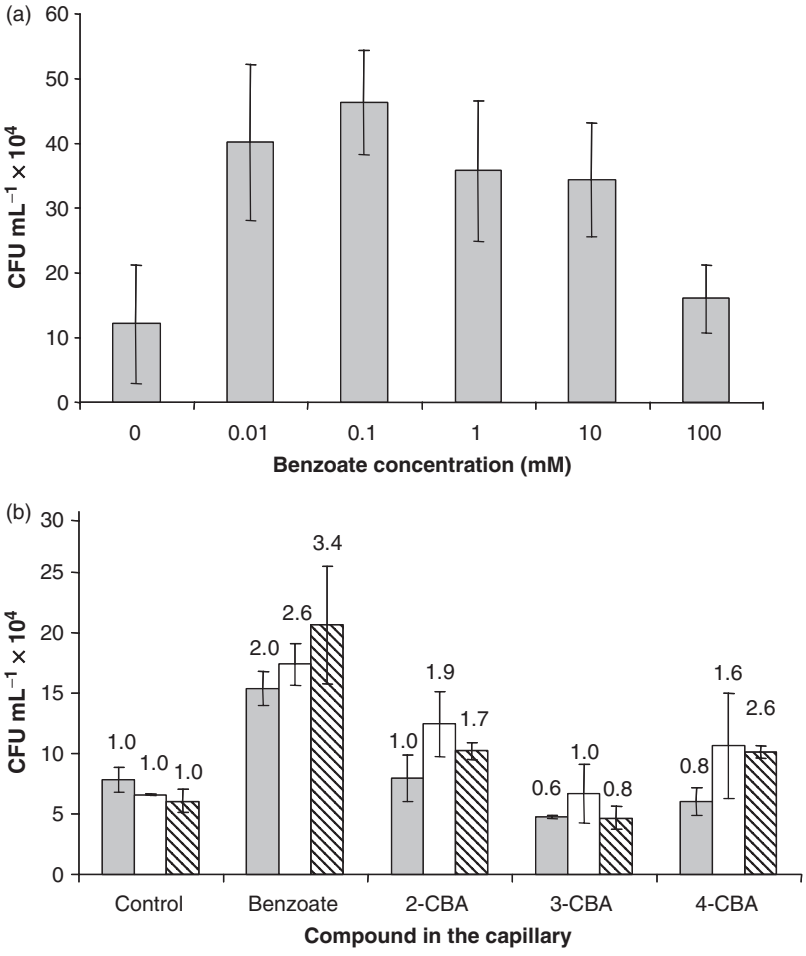

Fig. 4. Chemotactic response of *Pseudomonas* sp. B4 towards benzoate and chlorobenzoates. (a) Cells were grown to the mid-exponential phase in minimal medium containing 1% glucose as the sole carbon source. After centrifugation, the chemotactic response of *Pseudomonas* sp. B4 towards increasing benzoate concentration was examined. (b) Effect of growth medium in chemotaxis of *Pseudomonas* sp. B4 toward different CBAs. Cells were grown to the mid-exponential phase in minimal medium containing 1% glucose (grey bars), 0.1% biphenyl (white bars) or 0.1% benzoate (hatched bars) as the sole carbon sources. The capillary assay was done as in (a). The bacterial cells attracted by the indicated compounds were determined after duplicate plating in LB medium. Error bars indicate the SDs based on three different replicated experimental values. Numbers on top of each bar indicate the relative chemotactic indexes.

Cells were attracted to glucose and biphenyl when previously grown in defined media. Conversely, cells previously grown in rich medium (LB) did not show significant chemotactic responses (Fig. 2b). This may be due to the presence of an excess of reserve nutrients that prevent the chemical attraction by the tested carbon sources.

Capillary assays showed that *Pseudomonas* sp. B4 cells accumulated in capillaries containing 1 mM of biphenyl, 2-CB, 3-CB, 4-CB and 2,3-CB with RCI values higher than 2 in most cases (Fig. 3), confirming in general the results obtained by swarming towards these compounds (Fig. 1). In the case of 4-CB and 2,3-CB, the RCI values were not always higher than 2, probably due to their high toxicity and lower solubility in the chemotaxis buffer.

During biodegradation of PCBs, different chlorobenzoates are generated as intermediates (Potrawfke *et al.*, 1998). Some of these compounds are toxic to the biodegrading bacteria (Camara *et al.*, 2004; Parnell *et al.*, 2006) and therefore it was considered of importance to determine whether *Pseudomonas* sp. B4 was chemotactically stimulated by these derivatives. Figure 4a shows the chemotactic response to benzoate, reaching a maximum number of cells inside the capillary when the concentration was 0.1 mM, a value similar to that reported for other microorganisms (Harwood *et al.*, 1990). Cells previously grown in glucose (grey bars, Fig. 4b) showed a chemotactic effect to benzoate but not to its chloroderivatives. On the other hand, cells previously grown on biphenyl (Fig. 4b, white bars) or benzoate (Fig. 4b, hatched bars) were chemotactic towards benzoate but not its chloroderivatives. It is clear that previous growth of the cells on biphenyl or benzoate stimulated the chemotactic response of *Pseudomonas* sp. B4 towards benzoate, suggesting an inducible type of response. Previous growth on benzoate showed a stimulation of chemotaxis of the cells to 4-CBA. This effect was not seen in the case of 3-CBA, most likely due to a toxic effect of 3-CBA on the cells (see below). *Pseudomonas* sp. B4 used as growth substrates biphenyl, 2-CB, 3-CB, 4-CB and 2,3-CB but it did not grow on 2-CBA, 3-CBA and 4-CBA. 4-CBA was an attractant in spite of the lack of growth of *Pseudomonas* sp. B4 on it, suggesting that the response to this compound was not an energy taxis response.

Figure 5a shows that *Pseudomonas* sp. B4 has a chemotactic swarming response towards benzoate, being capable of

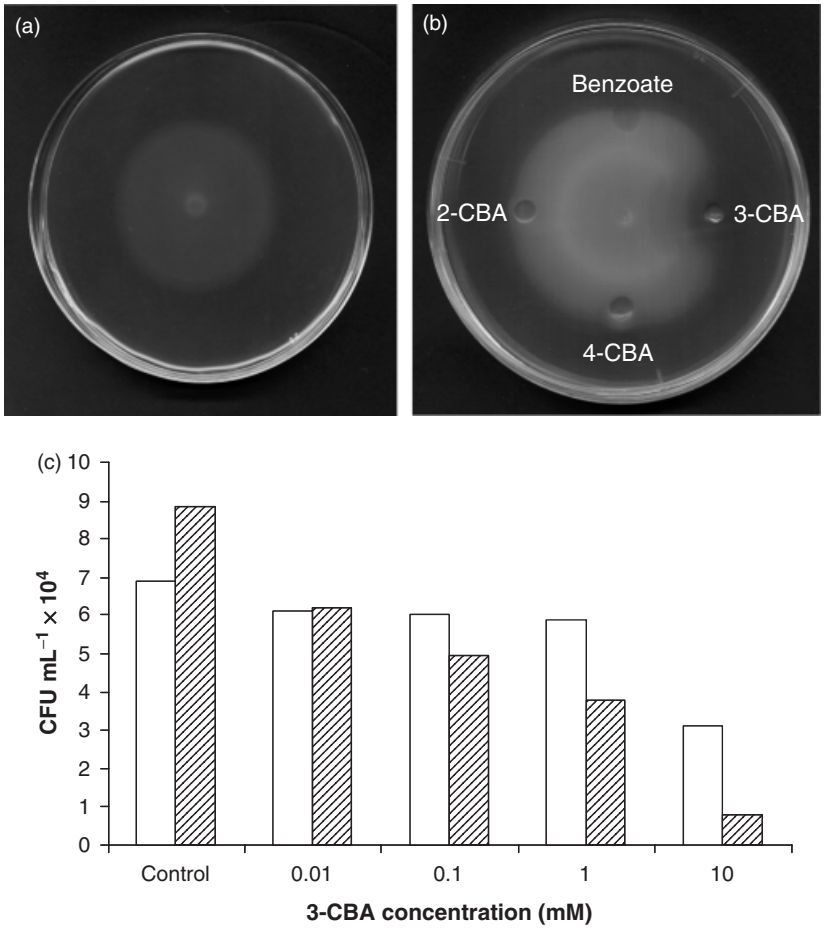

Fig. 5. Chemotactic swarming responses and toxic effect of 3-chlorobenzoate in *Pseudomonas* sp. B4. Cells grown in minimal medium were inoculated in plates containing the same medium supplemented with 1 mm benzoate (a) as the sole carbon source by stabbing them at a point corresponding to the center of the swarm ring and were incubated for 72 h at 30 °C. (b) Toxic effect of different CBAs in a swarming assay combined with a chemical-in-plug method. Cells grown in minimal medium were inoculated in plates containing the same medium supplemented with 1 mm benzoate as the sole carbon source as in (a), except that agarose plugs containing 100 mm of each of the indicated compounds were present on the plate at the beginning of the incubation. (c) Effect of different 3-CBA concentrations on cell viability of *Pseudomonas* sp. B4. Exponential phase suspensions of *Pseudomonas* sp. B4 previously grown in 1% glucose were exposed to different concentrations of 3-CBA and samples were taken at 0 (white bars) and 90 min (hatched bars) to determine the number of CFU.

metabolizing the attractant compound. No swarming assay was done with 3-chlorobenzoate as *Pseudomonas* sp. B4 did not grow on it and this assay requires that cells grow on the attractant to obtain a response. We explored the toxic effect of these chlorinated benzoates and found only 3-CBA to be toxic at a concentration of 0.1 mM (Fig. 5b and c). Nevertheless, we cannot exclude the possibility that 3-CBA may also be a repellent for *Pseudomonas* sp. B4. On the other hand, 2-CBA and 4-CBA did not interfere with the swarming in a benzoate-supplemented plate.

Chemotaxis toward benzoate and chlorobenzoates has been studied before in *P. putida* PRS2000 (Harwood *et al*., 1990). *Pseudomonas* sp. B4 behaved similarly to *P. putida* PRS2000 when confronted with benzoate (attractant in both microorganisms) or to 2-CBA (nonattractant in both cases). On the other hand, both *Pseudomonas* strains responded differently towards 3-CBA. The capacity of a compound to elicit a different chemotactic response is sometimes related to its nutritional properties. The same compound can act as an attractant for a microorganism capable of utilizing it as a growth substrate or as a repellent for a bacterium for which the compound is toxic. 3-CBA was very toxic for *Pseudomonas* sp. B4 and it could not grow on it (Fig. 5). In contrast, *P. putida* PRS2000 can grow on 3-CBA and it is an attractant for this bacterium (Harwood *et al*., 1990).

Synchronized regulation of bacterial chemotaxis to many xenobiotic compounds, with their respective degradation and/or transformation, indicates that this phenomenon might be an integral feature for degradation (Pandey & Jain, 2002; Chávez *et al*., 2006). It is clear that the efficiency of bioremediation of a contaminated field with organochlorine compounds will depend not only on the metabolic capacities and microbial chemotactic behaviour but also on the toxicity of the intermediates formed during the process and the commensal relationships with other members of the microbial consortium that is able to degrade these intermediates. For example, in a model consortium consisting of two organisms, *Burkholderia* sp. LB400 (nonmotile) and *Pseudomonas* sp. B13(FR1) (motile), the organisms apparently interact metabolically because *Pseudomonas* sp. B13(FR1) can reach and metabolize the CBA produced by *Burkholderia* sp. LB400 when grown on chlorobiphenyl (Nielsen *et al*., 2000).

We can conclude that PCB-degrading microorganisms to be used for bioremediation of soils contaminated with these compounds should have the capacity to detect and move through the soil particles towards a gradient of the PCB to be metabolized, having this way a more efficient microbe–substrate interaction. Conversely, nonmotile strains of PCB-degraders, such as the model strain *B. xenovorans* LB400, would be at a clear disadvantage to access these soil-sorbed organic pollutants.

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