The Dual Functions of Biphenyl-degrading Ability of Pseudomonas sp. KKS102: Energy Acquisition and Substrate Detoxification

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Received April 24, 2003; Accepted June 9, 2003

The bph operon of Pseudomonas sp. KKS102 is constituted of 11 bph genes which encode enzymes for biphenyl assimilation. Growth of a mutant in which a large part of the bph operon was deleted was inhibited by biphenyl in a concentration-dependent manner. We constructed a series of bph operon deletion mutants and tested for their biphenyl sensitivity. Growth inhibition by biphenyl was more prominent with the mutants defective in bphA1, bphB, bphC, and bphD, which were clustered in the bph operon and working in the early stage of the biphenyl degradation. The mutant defective in bphE, which was working at the late stage and forming a different cluster from the early stage genes, was not much inhibited by biphenyl. These indicate that biphenyl is detoxified by enzymes which function in the early stage of biphenyl assimilation and thus detoxification of substrates as well as energy acquisition could have played an important role in the evolution of the KKS102 bph operon.

Key words: Pseudomonas sp. KKS102; biphenyl; growth inhibition; detoxification

It is widely known that hydrocarbons such as aromatics, cycloalkanes, and terpenes are toxic to microorganisms. These lipophilic compounds tend to accumulate in the cell membrane, disorganizing its structure and impairing its vital functions.1-3 The toxicity of these compounds to microorganisms is very important from the viewpoint of microbial degradation of hydrocarbons, in which one of the major problems is low stability of the desired activity due to the inactivation of cells by the toxic substrates.

Polychlorinated biphenyls (PCBs) are stable hydrophobic compounds that are composed of biphenyl molecules containing variable numbers of chlorine and causing environmental problems because of their toxicity to human health. Most microorganisms that are able to degrade PCBs have a series of inducible genes encoding enzymes that function for their degradation.2-5 Before full expression of those genes, such microorganisms need to be exposed to biphenyl. To date, however, there have been only a few studies that have examined the effects of biphenyl or PCBs on a PCB-degrader. In general, it is known that microorganisms adapt to hydrocarbons by modifying their membranes (e.g., increase in lipid ordering, change in lipid/protein ratio) to maintain them in a fluid state.6-9 In the case of Ralstonia eutropha H850, a PCB-degrading bacterium, biphenyl and PCB increased the ratio of saturated to unsaturated fatty acids in the cell membrane.3 This change should counteract the disordering effects of biphenyl and PCBs, and at the same time, suggests that biphenyl and PCBs are partitioned in the cytoplasmic membrane.

In this study, we focused on the toxic effects of biphenyl on the growth of Pseudomonas sp. KKS102, a Gram-negative bacterium, which can use biphenyl as a sole source of carbon and energy and co-metabolize PCBs by biphenyl-metabolic enzymes. Up to now, the biphenyl/PCB degradation pathway and the genes responsible for biphenyl/PCB degradation have been well characterized and it has been shown that the bph operon is composed of 13 cistrons, bphEGF(orf4)A1A2A3BCD(orf1)A4R (Fig. 1).4-9 From a comparison with operons of other bacteria,3-9 this KKS102 bph operon seems to be evolved by fusion of three separate segments, bphABC, bphD, and bphEGF. The transcription of the bph operon starts from the pE promoter that is located in the upstream region of the bphE gene and is negatively regulated by BphS.9 The repression mediated by BphS protein is counteracted by a meta-cleaved metabolic intermediate of biphenyl, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA).10

Here we demonstrate that the biphenyl-degrading
ability of KKS102 enables them to survive in the presence of biphenyl by detoxifying it and confers the tolerance to biphenyl on KKS102.

Materials and Methods

Strains and media. Pseudomonas sp. KKS102 was used as a wild-type strain. KKS102 and its derivatives were grown and maintained at 30 °C on three-fold-diluted Luria-Bertani (1/3LB) medium (0.3% tryptone, 0.16% yeast extract, 0.5% NaCl) with or without biphenyl. Kanamycin (5 mg/l) was added, if necessary. For addition of biphenyl, biphenyl dissolved in 100 μl hexadecane was used. We confirmed that 100 μl hexadecane added to a culture did not impair the growth of KKS102 or its derivatives. Escherichia coli JM109 (recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, (lac-proAB)/F traD36, proAB, lacI, lacZΔM15) was used for plasmid preparations.

Construction of mutants. We disrupted the bph genes with the kanamycin-resistance gene derived from Tn5. For the construction of a mutant in which a large part of the bph operon was deleted, the PCR-based strategy described by Nikawa and Kawabata11) was used. For the construction of a series of bph operon deletion mutants, we used the plasmids constructed previously.10) Each of the plasmids was linearized by an appropriate restriction enzyme, extracted with phenol-chloroform, ethanol-precipitated, dissolved in HPLC grade water, and introduced into KKS102 or ΔS by electroporation. The electroporation was done as previously reported.10) Each mutant was analyzed by Southern blot analysis by using the disrupted gene as a probe (data not shown).

Measurement of growth. Cell growth was monitored by measuring the optical density (OD) at 600 nm (Bio-photorecorder TN 1506, Advantec). The temperature was kept at 30°C and shaking speed was 30 rpm.

Northern blot analysis. Total RNA was prepared with Isogen (Nippon Gene, Japan). Hybridization and detection were done using digoxigenin-labeled DNA with a CSPD system (Roche Diagnostics, Germany), according to the provider’s protocol.

Preparation of a crude cell extract. Cells were cultivated in 10 ml of 1/3LB medium and harvested at stationary phase, washed, and suspended in 1 ml of sample buffer (50 mM potassium phosphate buffer (pH 8.0) containing 10% glycerol). The cells were broken by vortexing with the same volume of glass beads. The supernatant of two successive centrifugations, at 4,000 rpm for 5 min at 4°C and 12,000 rpm
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Fig. 2. Harmful Effects of Biphenyl on the Growth of a bph Gene Deletion Mutant.

KKS102 (open symbols) and a bph gene deletion mutant (Δbp; solid symbols) were grown in a 1/3LB medium, in the absence of biphenyl (control medium) and in the presence of biphenyl. The growth was measured as optical density (OD) at 600 nm. Biphenyl (BP) was added when the OD600 reached 0.4. Experiments were repeated three times and a result of a representative experiment is shown. Symbols: ○, KKS102 control; ▲, Δbp control; ●, Δbp + BP.

for 5 min at 4°C, was used as a cell extract. Protein concentration was measured using the Bio-Rad protein assay with bovine gamma globulin as a standard.

Assay of BphD activity. The BphD activity of the mutants was measured as previously reported.9) One unit of BphD activity was defined as 1 nmol of HOPDA cleavage per minute, using 19,800 at 434 nm as the molar extinction coefficient for HOPDA.12)

Results

Growth of a bph gene deletion mutant in the presence of biphenyl

We constructed a deletion mutant, designated Δbp, which lacks a large part of the bph operon from bphA1 to bphA4 and hence the ability to degrade biphenyl. After adding biphenyl to the bacterium in 1/3LB medium at OD600 = 0.4, the growth of Δbp was severely inhibited whereas the growth of KKS102 was first slow for 8 h and then, after adding biphenyl, accelerated to reach a far higher turbidity presumably because of the use of biphenyl as a carbon source (Fig. 2). Under the same conditions, colony forming units (CFU) were measured to monitor the cell viability. Viable cell counts were well correlated with the change of culture turbidity (OD600) (data not shown). Therefore, in this paper we have examined culture turbidity as an indicator of bacterial growth. The result in Fig. 2 shows that the strain KKS102 is sensitive to biphenyl unless it has an intact bph operon. This suggests that biphenyl degradation enables KKS102 to be tolerant to the toxicity of biphenyl.

Effects of high concentration of biphenyl on the growth of bph gene null mutants

To examine the relationship between the tolerance to biphenyl and the biphenyl-degrading ability of KKS102, we constructed five mutants with disruptions in each of one of five bph genes (bphA1, bphB, bphC, bphD, and bphE). Expecting the expression of intact bph genes without a bph operon inducer HOPDA, bphS gene, the product of which represses the expression of bph operon, was also deleted in these five mutants. The expression levels of the bph operon in these mutants were estimated by northern blot analysis on mRNA of bphD that is downstream from the bph operon and by measuring BphD activity (Fig. 3). It is quite likely that all the mutants expressed the bph genes at similar levels.

By adding a high concentration (14,000 ppm) of biphenyl to the culture medium, growth of the mutant defective in bphA1, bphB, or bphC was stopped immediately. On the other hand, the growth of mutants defective in bphD or bphE was less in-
Effects of a High Concentration of Biphenyl (14,000 ppm) on the Growth of bph Gene Null Mutants.

The culture conditions are the same as those described in Fig. 1. Symbols: ○, ΔS; △, ΔSΔA; □, ΔSΔB; ◊, ΔSΔC; ×, ΔSΔD; −, ΔSΔE.

Fig. 4.

Effects of Lower Concentration of Biphenyl (80 ppm) on the Growth of bph Gene Null Mutants.

The culture conditions are the same as described in Fig. 1 and symbols in the figure are the same as those described in Fig. 4. Symbols: ○, ΔS; △, ΔSΔA; □, ΔSΔB; ◊, ΔSΔC; ×, ΔSΔD; −, ΔSΔE.

Fig. 5.

Growth inhibition by biphenyl and its metabolites

It is generally accepted that intermediate metabolites of aromatic compounds could be more toxic than the original substrates. To evaluate the toxicity of biphenyl metabolites to Δbp, we added 2,3-dihydroxybiphenyl (2,3-DHBP) or HOPDA at the same molar concentration as biphenyl to the culture medium. As shown in Fig. 6, 2,3-DHBP was less inhibitory to the growth than biphenyl. HOPDA was not inhibitory at all. This result suggests that biphenyl is detoxified through the early stage of its degradation and thus KKS102 is tolerant to biphenyl.

Fig. 6.

Discussion

In this report we showed that a bph gene deletion mutant which lacked the biphenyl-degrading ability was sensitive to biphenyl. We also demonstrated that a toxic effect of biphenyl was relieved through biphenyl assimilation. However, the toxic mechanism of biphenyl in KKS102 remains uncertain. Biphenyl has been widely used in organic syntheses, heat transfer fluids, dye carriers, food preservatives, and as a fungistat in the packaging of citrus fruits. The toxic effects of biphenyl to a variety of living things have been evaluated by many researchers and growth inhibitory effect of biphenyl has been reported with some kinds of plants, fungi, and aquatic life forms.13,14) In this paper, we observed inhibitory effects of biphenyl on the biphenyl degradation-deficient mutants of Pseudomonas sp. KKS102. It has been shown that the fluidizing effect of hydrophobic compounds on membranes, which increase the passive influx of proton, leads to cellular growth inhibition.15) In addition, it has been shown that biphenyl accumulates in the cytoplasmic membrane.16) Therefore, the observed growth impairment by biphenyl may have resulted from its accumulation.
in the cytoplasmic membrane, which should cause reduced barrier function of the membrane.

We evaluated the toxic effects of biphenyl and its intermediate metabolites, 2,3-DHBP and HOPDA. Both 2,3-DHBP and HOPDA affected growth of the strain Δbp less, indicating that these compounds are not very harmful to growth. This may be because of their lower hydrophobicity. Thus it is considered that biphenyl is detoxified along with its degradation and the reduction of hydrophobicity. On the other hand, the toxicity of hydroxy-biphenyl has been reported in a PCB-degrading bacterium, *Comamonas testosteroni* TK102,\(^1\)\(^6\) where monohydroxybiphenyls and monohydroxy-PCBs, which were produced spontaneously from cis-2,3-dihydrodiol derivatives of biphenyl and PCBs, products of initial dioxygenation, inhibited bacterial cell separation. It has also been shown that monohydroxybiphenyls inhibited cell separation of some other Gram-negative bacteria.\(^1\)^\(^6\) Monohydroxybiphenyls or monohydroxy-PCBs are more hydrophobic than their dihydroxy form and hence should be more inhibitory to membrane functions.

Here we demonstrated that the degrading ability of biphenyl makes *Pseudomonas* sp. KKS102 tolerant to the substrate. In general, the advantage of the acquisition of hydrocarbon-degrading abilities is that microorganisms can use these compounds as an energy and carbon source and thus these creatures become more competitive in the environment. In addition to this, it is possible that microorganisms may acquire hydrocarbon-degrading ability in order to detoxify these hazardous chemicals and survive a severe environment. There are some studies about the correlation between detoxification and degradation. For example, it is reported that the imperfect fungus *Paecilomyces lilacinus* has a high transformation capacity for biphenyl and this could explain this organism’s tolerance to this fungicide.\(^1\)^\(^7\)

Generally, the *bph* operon is considered to be composed of early pathway genes (*bphABC*) and late pathway genes (*bphEFG*).\(^2\)\(^3\) It is suggested that there is a promoter-like region between late pathway genes and the early pathway genes in the *bph* operon of KKS102 (unpublished data). Moreover, the *bphS* gene is divergently oriented in the upstream region of *bphE* and separated from *bphE* by an insertion sequence.\(^3\)\(^5\) Thus, from these observations, it could be assumed that the early pathway genes originally functioned for detoxification of substrates and later they were combined with the late pathway genes and evolved to form the *bph* operon in KKS102, which is regulated by BphS. Detoxification of biphenyl as well as energy and carbon acquisition could be a driving force for evolution of the KKS102 *bph* operon.

**Acknowledgment**

We are grateful to Ms Setsu Yamagami for her helpful discussions.

**References**


