Research Article

Diversity of 4-Chloro-2-nitrophenol-Degrading Bacteria in a Waste Water Sample

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Eighteen bacterial strains, isolated from a waste water sample collected from a chemically contaminated site, Patancheru (17°32'N 78°16'E/17.53°N 78.27°E), India, were able to decolorize 4-chloro-2-nitrophenol (4C2NP) in the presence of an additional carbon source. These eighteen 4C2NP-decolorizing strains have been identified as members of four different genera, including *Bacillus*, *Paenibacillus*, *Pseudomonas*, and *Leuconostoc* based on the 16S rRNA gene sequencing and phylogenetic analysis. Most of the bacteria (10) belonged to the genus *Bacillus* and contributed 56% of the total 4C2NP-degrading bacteria, whereas the members of genera *Paenibacillus* and *Pseudomonas* represented 22% and 17%, respectively, of total 4C2NP-degrading isolates. There was only one species of *Leuconostoc* capable of degrading 4C2NP. This is the first report of the diversity of 4C2NP-decolorizing bacteria in a waste water sample. Furthermore, one bacterium, *Bacillus aryabhatai* strain PC-7, was able to decolorize 4C2NP up to a concentration of 2.0 mM. Gas chromatography-mass spectrometry analysis identified 5-chloro-2-methylbenzoxazole as the final product of 4C2NP decolorization in strain PC-7.

1. Introduction

4-Chloro-2-nitrophenol (4C2NP) is a toxic compound that is widespread in the environment including ground water and soil [1]. Both physiochemical and biological methods have been used for 4C2NP decontamination [1–4]. Biological methods have advantages over physiochemical methods because they are more effective and less expensive. Among biological methods, bacterial remediation is an effective way to remove xenobiotic compounds [1].

Few bacteria capable of utilizing 4C2NP as their sole sources of carbon and energy have been isolated and characterized from a variety of sources [5, 6]. A 4C2NP-mineralizing bacterium, *Exiguobacterium* sp. PMA, initiates the 4C2NP degradation with reduction of 4C2NP into 4-chloro-2-aminophenol that is then dehalogenated to aminophenol with release of chloride ion. Further degradation of aminophenol proceeds via ammonia release [5]. A genetically engineered bacterium, *Pseudomonas* sp. N31, was also capable of degrading 4C2NP via chloride and nitrite

releases and the formation of 4-chlorocatechol [6]. A mixed culture of *Alcaligenes* sp. and *Enterobacter* sp. degraded 4C2NP via 4-chloro-2-aminophenol [7].

Few bacteria biotransformed toxic 4C2NP into other less toxic compounds rather than performing its complete mineralization [8–10]. A marine bacterium, *Bacillus* sp. MW-1 [8], a soil bacterium, *Bacillus subtilis* RKJ 800 [9], and *Pseudomonas* sp. JHN [10] decolorized and biotransformed 4C2NP into 5-chloro-2-methylbenzoxazole. In this transformation mechanism, 4-chloro-2-aminophenol and 4-chloro-2-acetaminophenol were also detected as metabolites.

The aim of this study is to identify and characterize additional 4C2NP-degrading bacteria. In this communication, we have studied the diversity of 4C2NP-degrading bacteria.

2. Materials and Methods

2.1. Isolation of 4C2NP-Degrading Bacteria. Eighteen bacterial strains were isolated from a waste water sample collected from a chemically contaminated site, Patancheru (17°32'N

FIGURE 1: Phylogenetic tree of 4-chloro-2-nitrophenol-decolorizing bacteria based on their 16S rRNA genes.

78°16'E/17.53°N 78.27°E), India, by an enrichment method using 4C2NP as a cosubstrate. Patancheru is the major industrial hub of Andhra Pradesh, India, where several major chemical industries are located. This place has been considered one of the worst polluted places in the world [11, 12]. The highest level of drug pollution in water was found in Patancheru in 2009 [12]. Researchers have observed measurable quantities of 21 different manufactured drugs in water [12]. This pollution results from waste water dumped into the river by the over 90 local pharmaceutical manufacturers [12].

For enrichment, 1 mL of the waste water sample was added to a 200 mL Erlenmeyer flask containing 50 mL minimal media, 0.5 mM 4C2NP, and 10 mM glucose. The minimal medium was prepared by dissolving the following compounds in 100 mL of double distilled water: 0.4 g Na_2HPO_4 , 0.2 g KH_2PO_4 , 0.08 g NaNO_3 , 0.08 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 mL trace element solution, and 1.8 g agar [5]. One liter of the trace element solution contained 0.10 g $\text{Al}(\text{OH})_3$, 0.05 g $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05 g KI, 0.05 g LiCl, 0.08 g MgSO_4 , 0.05 g H_3BO_3 , 0.10 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g CoCl_2 , 0.01 g $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$, 0.05 g BaCl_2 , and 0.05 g $(\text{NH}_4)_6$

$\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ [5]. The enrichment flask was incubated at 30°C under shaking conditions at 200 rpm. Upon decolorization of the yellow color of 4C2NP in the flask, the culture was serially diluted and plated on minimal agar plates containing 0.5 mM 4C2NP and 10 mM glucose. Eighteen different morphotypes were grown on the plates containing 4C2NP and 10 mM glucose. Eighteen colonies showed decolorization among them suggesting that these bacteria have the ability to utilize 4C2NP. These colonies were streaked further on nutrient agar plate to check the purity and pure cultures were used to monitor their ability to degrade 4C2NP at higher concentrations (from 0.5 to 2.5 mM).

2.2. Identification of 4-Chloro-2-nitrophenol-Decolorizing Bacteria and Phylogenetic Analysis. All 4C2NP-decolorizing bacteria were identified on the basis of 16S rRNA gene sequencing using the universal primers 27f and 1492r as described previously [13, 14]. The 16S rRNA gene sequences of related strains were retrieved from the NCBI and aligned using the ClusterW program. A phylogenetic tree was

TABLE 1: Showing degradation of various concentrations of 4-chloro-2-nitrophenol by the eighteen bacteria.

Serial number	Strain	Proposed bacterial name based on the 16S rRNA gene sequencing	Isolation source	The 4C2NP concentration where strain can be completely transformed
1	Strain PC-1	<i>Bacillus cereus</i>	Contaminated soil	1.0 mM
2	Strain PC-2	<i>Bacillus toyonensis</i>	Contaminated soil	0.9 mM
3	Strain PC-3	<i>Bacillus thuringiensis</i>	Contaminated soil	1.0 mM
4	Strain PC-4	<i>Bacillus firmus</i>	Contaminated soil	0.8 mM
5	Strain PC-5	<i>Bacillus koreensis</i>	Contaminated soil	0.6 mM
6	Strain PC-6	<i>Bacillus megaterium</i>	Contaminated soil	1.5 mM
7	Strain PC-7	<i>Bacillus aryabhattai</i>	Contaminated soil	2.0 mM
8	Strain PC-8	<i>Bacillus aerophilus</i>	Contaminated soil	0.6 mM
9	Strain PC-9	<i>Bacillus siamensis</i>	Contaminated soil	0.8 mM
10	Strain PC-10	<i>Bacillus amyloliquefaciens</i>	Contaminated soil	0.9 mM
11	Strain PC-11	<i>Paenibacillus massiliensis</i>	Contaminated soil	0.7 mM
12	Strain PC-12	<i>Paenibacillus kribensis</i>	Contaminated soil	0.6 mM
13	Strain PC-13	<i>Paenibacillus kribensis</i>	Contaminated soil	0.5 mM
14	Strain PC-14	<i>Paenibacillus terrae</i>	Contaminated soil	0.5 mM
15	Strain PC-15	<i>Leuconostoc citreum</i>	Contaminated soil	0.5 mM
16	Strain PC-16	<i>Pseudomonas lini</i>	Contaminated soil	0.8 mM
17	Strain PC-17	<i>Pseudomonas palleroniana</i>	Contaminated soil	0.9 mM
18	Strain PC-18	<i>Pseudomonas extremorientalis</i>	Contaminated soil	0.6 mM

constructed using the neighbour-joining method as implemented in MEGA [13, 14].

2.3. Growth and Decolorization Studies. Bacterial growth was measured in 250 mL minimal medium containing 10 mM glucose and various concentrations of 4C2NP (0.5, 1.0, 1.5, 2.0, and 2.5 mM). At every 2 h, 3 mL samples were collected; 1 mL sample was used for measuring the growth at 600 nm using a spectrophotometer; another 2 mL sample was centrifuged and the supernatant was used to monitor the 4C2NP decolorization as well as chloride, ammonia, and nitrite release. The 4C2NP decolorization was measured as the decrease in the optical density at 400 nm and the % decolorization was calculated as described previously [8–10]. The chloride, nitrite, and ammonia ions were monitored as described previously [5].

2.4. Identification of Metabolites. Bacterial strains were grown on minimal media containing 10 mM glucose and 2.0 mM 4C2NP; samples were collected at regular intervals and extracted with ethyl acetate and the extracted samples were analyzed by gas chromatography-mass spectrometry using an Agilent Gas Chromatography system equipped with Time-of-Flight Mass Spectrometer and HP-5 column as described previously [10]. Helium was used as carrier gas at 1.5 mL/min. The initial column temperature was held at 50°C for 1 min and then temperature was increased at 25°/min to 290°C and held for 10 min. The samples (1 µL) were injected in splitless mode. The temperatures of the transfer line and ion source (electron ionisation mode, EI, 70 eV) were 225°C and 250°C, respectively.

3. Results and Discussion

Eighteen bacterial strains, isolated from a chemically contaminated waste water sample collected from Patancheru, decolorized 4C2NP in the presence of glucose. The analysis of the 16S rRNA gene sequences of these strains showed that 10 bacterial strains designated as strain PC-1 to PC-10 belonged to the genus *Bacillus* whereas 4 bacterial strains, designated as strain PC-11 to PC-14, belonged to the genus *Paenibacillus*. Strain PC-16, strain PC-17, and strain PC-18 belonged to genus *Pseudomonas* and strain PC-15 to the genus *Leuconostoc*.

Results of the 16S rRNA gene sequencing of all eighteen strains were also supported by phylogenetic analysis. Phylogenetic analysis of all eighteen strains with closely related species showed that these strains are distinct to each other and belong to four different genera, that is, *Bacillus*, *Pseudomonas*, *Paenibacillus*, and *Leuconostoc* (Figure 1).

Figure 2 represents the diversity of 4C2NP-degrading bacteria among these eighteen strains. It was observed that *Bacillus* spp. are dominant for decolorization of 4C2NP and contributed 56% of the total 4C2NP-degrading bacteria, whereas *Paenibacillus* spp. and *Pseudomonas* spp. represented 22% and 17%, respectively, of total 4C2NP-degrading bacteria. There was only one species of *Leuconostoc* capable of degrading 4C2NP, representing 5.0% of the diversity.

We have also investigated decolorization of 4C2NP at various concentrations by these eighteen strains and the results are summarized in Table 1. They showed that *Bacillus aryabhattai* strain PC-7 was able to decolorize 4C2NP up to 2.0 mM concentration. This strain was selected for detailed study.

FIGURE 2: Diversity of 4-chloro-2-nitrophenol-decolorizing bacteria.

FIGURE 3: Growth of *Bacillus aryabhattai* strain PC-7 on minimal medium containing 10 mM glucose and (a) 0.5 mM 4-chloro-2-nitrophenol, (b) 1.0 mM 4-chloro-2-nitrophenol, (c) 1.5 mM 4-chloro-2-nitrophenol, (d) 2.0 mM 4-chloro-2-nitrophenol, and (e) 2.5 mM 4-chloro-2-nitrophenol.

Bacillus aryabhattai strain PC-7 was grown in minimal medium containing 10 mM glucose in the presence of different concentration of 4C2NP (0.5, 1.0, 1.5, 2.0, and 2.5 mM). As the 4C2NP concentration was increased, the growth of strain PC-7 was decreased. There was no growth with 2.5 mM 4C2NP. The optimum growth was observed at the concentration of 0.5 mM, whereas minimum growth was observed at 2.0 mM concentration (Figure 3). The 4C2NP decolorization was also monitored at various concentrations and it was observed that strain PC-7 decolorized 0.5 mM 4C2NP very quickly (in only 12 hours), whereas it took 24 h to decolorize the 2.0 mM concentration (Figure 4). There was

FIGURE 4: Decolorization of 4-chloro-2-nitrophenol by *Bacillus aryabhattai* strain PC-7. (a) 0.5 mM 4-chloro-2-nitrophenol, (b) 1.0 mM 4-chloro-2-nitrophenol, (c) 1.5 mM 4-chloro-2-nitrophenol, (d) 2.0 mM 4-chloro-2-nitrophenol, and (e) 2.5 mM 4-chloro-2-nitrophenol.

FIGURE 5: Mass spectrum of the decolorized product of 4-chloro-2-nitrophenol by *Bacillus aryabhattai* strain PC-7.

no decolorization at the concentration of 2.5 mM because strain PC-7 was unable to grow in these conditions.

GC-MS was carried out to identify the product of 4C2NP decolorization. The mass spectrum of the final metabolite contains a molecular ion peak at m/z 167 and other fragments are observed at m/z 104 and m/z 63 (Figure 5). On the basis of NIST library search, this product showed 92.4% similarity between its mass spectrum and the mass spectrum of 5-chloro-2-methylbenzoxazole. To date, few bacteria are known to degrade 4C2NP, which include *Bacillus* sp. MW-1 [8], *Bacillus subtilis* RKJ 700 [9], *Pseudomonas* sp. JHN [10], *Pseudomonas* sp. N31 [6], *Exiguobacterium* sp. PMA [5], and a mixed culture of *Alcaligenes* sp. and *Enterobacter* sp. [7]. In this study, we have characterized eighteen bacterial strains isolated from a contaminated water sample of India that are able to degrade 4C2NP. These bacteria belong to four different genera including *Pseudomonas*, *Bacillus*, *Leuconostoc*, and *Paenibacillus* and they decolorized 4C2NP only in the presence of an additional carbon source. All eighteen bacterial strains were able to decolorize 4C2NP up to various concentrations. Previous studies showed that *Bacillus* sp. MW-1 was able to degrade 4C2NP at the concentration of 0.3 mM [8]; *Pseudomonas* sp. JHN was able to decolorize 0.5 mM concentration [10] and strain RKJ 700 was

FIGURE 6: Biotransformation of 4-chloro-2-nitrophenol to 5-chloro-2-methylbenzoxazole.

able to decolorize the concentration of 1.5 mM [9]. In this study, *Bacillus aryabhatai* strain PC-7 was able to decolorize 4C2NP up to 2.0 mM (the highest concentration reached thus far).

Bacterial degradation of 4C2NP proceeds via either mineralization or biotransformation. Few bacteria mineralized 4C2NP completely without requirement of any additional carbon source [5]. *Exiguobacterium* sp. PMA mineralized 4C2NP via 4-chloroaminophenol and aminophenol [5]. The 4C2NP biotransformation involves the conversion of 4C2NP into nontoxic compounds via detoxification. *Bacillus* sp. MW-1 and *B. subtilis* RKJ 700 biotransformed 4C2NP into 5-chloro-2-methylbenzoxazole. In this study, *Bacillus aryabhatai* strain PC-7 also biotransformed 4C2NP into 5-chloro-2-methylbenzoxazole (Figure 6). However, strain PC-7 was able to decolorize a high concentration of 4C2NP that was not previously reported for any bacterial strain.

4. Conclusion

Eighteen bacterial strains belonging to four different genera, including *Bacillus*, *Paenibacillus*, *Pseudomonas*, and *Leuconostoc*, decolorized 4C2NP in the presence of glucose. *Bacillus aryabhatai* PC-7 is an efficient degrader and decolorized 4C2NP up to 2.0 mM concentrations. 5-Chloro-2-methylbenzoxazole was identified as the final product of the 4C2NP decolorization.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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