Bull. Environ. Contam. Toxicol. (2004) 73:666–673

DOI: 10.1007/s00128-004-0478-x

Bulletin of Environmental Contamination and Toxicology

Detection of Chlorinated Phenols in Kraft Pulp Bleaching Effluents Using DmpR Mutant Strains

V. L. Campos,[1] C. A. Zaror,[2] M. A. Mondaca[1]

[1] Microbiology Department, University of Concepción, Post Office Box 160-C, Correo 3, Concepción, Chile
[2] Chemical Engineering Department, University of Concepción, Post Office Box 53-C, Correo 3, Concepción, Chile

Received: 12 March 2004/Accepted: 15 July 2004

Chlorinated phenols and their derivatives are well known by their high toxicity and may produce negative impact when released to the environment (Fleming 1995). Such compounds are commonly generated during cellulose pulp bleaching, and strict controls should be implemented to limit their discharge into water courses. For instance, the U.S. Environmental Protection Agency, published norms aimed at controlling organochlorine emissions from cellulose pulp bleaching, such as 2-chlorophenol, 2,4-dichlorophenol, 2.4.6-trichlorophenol, 4-chloro-3-methylphenol, 2,4-dimethylphenol, and other priority pollutants (Federal Register 1998). Effective implementation of such controls implies the use of accurate analytical techniques capable of detecting the presence of such key pollutants in contaminated waters and soils. However, the use of conventional analytical techniques based on spectrometry (UV, visible, IR) and chromatography (GC, TLC, HPLC), for routine detection and quantification of toxic pollutants is limited by high operational costs (Martinez et al. 1996; Scheper T 1992; Scheller and Shubert 1992). On the other hand, biosensors using genetically modified bacteria could be used as a cheaper and simpler alternative (Harayama and Timmis 1992). Such biosensors should contain a reporter gene, e.g., β-galactosidase (*lacZ*) or luciferase (*lux*) genes, which operates under transcriptional activator control. The presence of a key contaminant stimulate the transcriptional activator, expressing the reporter gene which, in turn, could be detected by the corresponding enzymatic activity. Thus, under appropriate conditions, a direct correlation between the contaminant concentration and the enzymatic activity could be found (Willardson et al. 1998; Rasmussen et al. 1997).

Various bacterial biosensors have been described in the literature (e.g. Heitzen et al. 1994; King et al. 1991; Burlage et al. 1994; Aizawa et al. 1995; Ikariyama et al. 1997; Willardson et al. 1998; De Lorenzo et al. 1993; Applegate et al. 1998). Recently, Wise and Kuske (2000) have obtained a set of mutant bacterial strains capable to detect phenolic compounds, such as: phenol, 2-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, pentachlorophenol, 4-chloro-3-metilphenol, 2,4-dimetilphenol, 2-nitrophenol, 4-nitrophenol, 2,4-dinitrophenol and 2-metil-4,6-dinotrophenol, all considered as priority pollutants by the US-EPA (Wise and Kuske 2000). These authors modified the phenol detection capacity of the DmpR protein by PCR mutagenesis in the DmpR sensor domain. Detection of phenolic

Correspondence to: M. A. Mondaca

compounds could be shown by activation of the *lacZ* gene transcription, producing a measurable signal (i.e. β-galactosidase activity).

It is interesting to know whether such biosensors could detect key pollution in real effluents, where complex mixture and toxic effects could impair bacterial activity. In this context, this paper reports experimental results on the capacity of three mutant DmpR strains to detect chlorinated organic compounds present in kraft pulp bleaching effluents.

MATERIALS AND METHODS

Bacterial strains, DmpR mutants B9, B23 and B24 were kindly provided by Dr Kuske (Environmental Molecular Biology Group, Biosciences Division, Los Alamos National Lab, New Mexico), were used.

Synthetic effluents were prepared by mixing various chlorinated phenols and non chlorinated aromatic compounds. Analytical grade chemicals assayed here included: phenol, 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,4-dichlorophenol, 4-chloro-3-methylphenol, 2,4-dimethylphenol, 2,4,6-trimethylphenol, 2,4,5-trimethylphenol, pentachlorophenol, toluene, xylene, benzene, 2-nitrotoluene, 2-methyl-4,6-dinitrophenol, and 2,4-dinitrophenol. All were obtained from Merck and, in each case, 25 mM stock solution in ethanol were prepared and stored for further use in sample preparations.

Kraft pulp bleaching effluents, unbleached kraft pulp from Chilean pinewood was provided by a local mill, featuring two levels of lignin content, viz. measured as kappa numbers 13 and 25. High kappa pulps yield bleaching effluents with a higher concentration of chlorinated organic compounds. Oven dried unbleached pulp samples (800 g) were bleached using a standard sequence. This featured a first stage treatment with 70% Cl_2 /30% ClO_2 (kappa factor 0.2, 5% consistency at 40°C for 40 min), followed by alkaline extraction in the presence of oxygen gas (3 bar pressure, 70°C, 10% consistency for 60 min). A final ClO_2 treatment was carried out to obtain a bleached pulp with 90°ISO brightness. At each stage, treated pulp was washed with a total volume of 16 dm^3 demineralised water. All bleaching wastewater were collected and stored at 2°C in a refrigerator, for further analysis and treatment. These bleaching effluents presented absorbable organic halogen (AOX) concentration levels around 22 (mg/l) and 7 (mg/l), corresponding to the high and low lignin content pulp, respectively.

For the detection of chlorinated phenols and other aromatic compounds, DmpR mutant strains were cultivated overnight in Luria-Bertani (LB) broth in presence of tetracycline (10.5 mg/mL), a 37° C, in a stirred bath. 800 µl cultured sample featuring an absorbance in the range 0.8-1.0 at 595 nm (A_{595}). Then, bacteria were pelletised by centrifugation and, resuspended in Luria-Bertani broth, in presence of solutions containing different concentrations of tested compounds, at 37°C during 2h, in a stirred bath. Bacterial cells were pelletised by centrifugation and refrigerated at –70°C. Similar experiments were conducted using raw and treated cellulose bleaching effluents, by resuspending 800 µl in the filtered effluent.

For determination of the biosensor stability in untreated kraft pulp bleaching effluent, bacterial cells were cultured in Luria broth at 35°C overnight. When cells reached an absorbance within 0.80 and 1.0 at 595 nm, 800-µl samples were pelletised by centrifugation, and immediately suspended in 800 µl bleaching effluents previously filtered using 0.2 µm micropore filters. Then, they were incubated in a stirred bath at 37°C, over a period of 24 h. Bacterial growth was monitored every 4 h using the microdrop technique (Bale et al.1988), and absorbance was determined using a Lambda Bio UV-visible spectrophotometer.

Liquid β-galactosidase assays were performed by using a modification of Miller's assay (Miller 1992). Cell sample pellets were thawed and suspended in 800 ml of Z buffer (60 mM Na_2HPO_4 H2O, 40 mM NaH_2PO_4 H_2O, 10 mM KCl, 1 mM $MgSO_4$ 7H2O). The absorbance at 595 nm of 100 ml of each cell suspension was determined in a microtiter plate by using an automated microplate reader (BIO-TEK Instruments, Inc., Winooski, Vt.). Following the addition of 15 ml of 0.1% (wt/vol) sodium dodecyl sulphate and 20 ml of $HCCl_3$, the remaining cell suspension was vortexed for 30 s to lyse cells, and 100 ml of each lysed sample was placed in the well of a microtiter plate. Each assay reaction was initiated with the addition of 50 ml of o-nitrophenyl-β-D-galactopyranoside (2.5 mg/ml). Reaction mixtures were incubated at 26°C, and reactions were stopped with the addition of 50 ml of 1M Na_2CO_3. Colour development for each reaction was detected by absorbance measurement at 415 nm on the microplate reader. Enzymatic activity was calculated as

$$\text{β-galactosidase activity} = \frac{1000 \times A_{415}}{\text{time} \times A_{595}}$$

where time is the reaction time in minutes, and A_{415} and A_{595} are absorbance measurements at 415 and 595 nm, respectively.

RESULTS AND DISCUSSION

The capacity to detect chlorinated phenolic compounds by mutant DmpR B9, B23 y B24 strains is shown in Table 1. All 9 assayed compounds induced a response from the three bacterial biosensors, when present in single solution at 100 µM concentration. Mutant DmpR strains presented the highest response in the presence of phenol and mono chlorinated phenols. Lower inductions were observed in the case of 3-chloro-4-methylphenol; 2,4-dimethylphenol; 2,4,6-triclorophenol and 2,4,5-triclorophenol. On the other hand, pentachlorophenol and 2-methyl-4,6-dinitrophenol were not detected by mutant DmpR strains. The low pk_a of pentachlorophenol (i.e. 4.9) and 2-methyl-4,6-dinitrophenol (i.e. 4.2) suggests that these molecules could not efficiently enter into the cell, due to deprotonation of hydroxyl groups at the induction medium neutral pH. Moreover, cellular lyses was observed at 2,4,6-trichlorophenol and pentachlorophenol concentrations above 75 µM and 50 µM, respectively. Assays with 2,4-

dinitrophenol showed a variable induction of enzymatic activity, and no response was induced at concentrations above 25 μM, in agreement with reports by Wise and Kuske (2000).

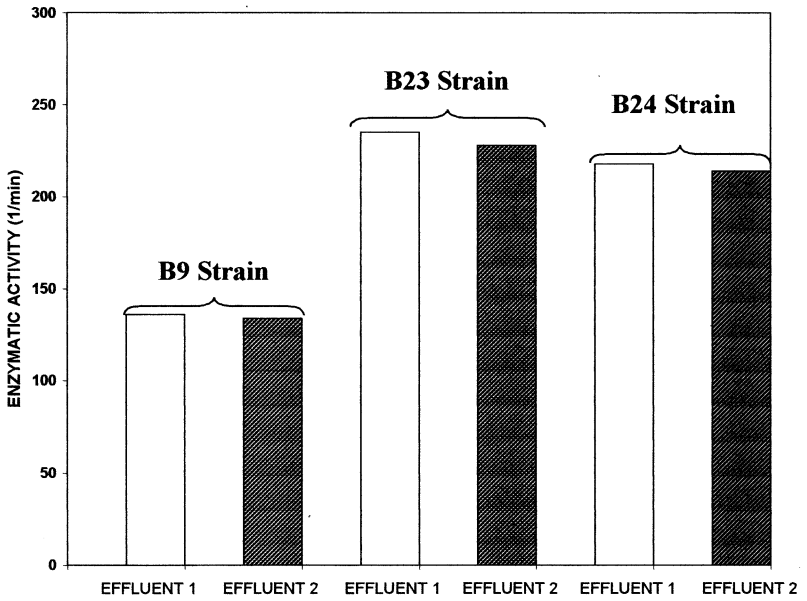

Figure 1. Response of mutant DmpR strains to synthetic effluents containing phenolic compounds (2 h culture at 37°C). "Effluent 1" contains a mixture of chlorinated phenols. "Effluent 2" contains the same mixture of chlorinated phenol, but in presence of non chlorinated aromatics.

In order to assess the mutant DmpR strains capacity to detect key pollutants in complex mixtures, assays were conducted using synthetic effluents. Figure 1 presents results of bacterial response to two synthetic effluents tested here: "Effluent 1" was composed of a mixture of 4-chloro 3-methyl phenol, 2,4-dichlorophenol, 4-chlorophenol, 3-chlorophenol, 2-chlorophenol, 2,4,6-trichlorophenol, and 2,4,5-trichlorophenol, at a concentration around 75 μM each. On the other hand, "Effluent 2" contained the same mixture of chlorinated phenols, but in presence of 2-nitrotoluene, xylene and benzene, all at 50 μM each. The three mutant DmpR strains showed enzymatic response. Moreover, B23 and B24 strains presented higher induced response than B9, in agreement with results reported in Table 1, for single compounds. Such results would indicate that mutant DmpR strains present high specificity, since toluene, xylene and 2-nitrotoluene are effectors of the XylR protein sensor domain and the sensor domain of XylR is 64% identical to DmpR at the amino acid composition (Shingler and Moore 1994).

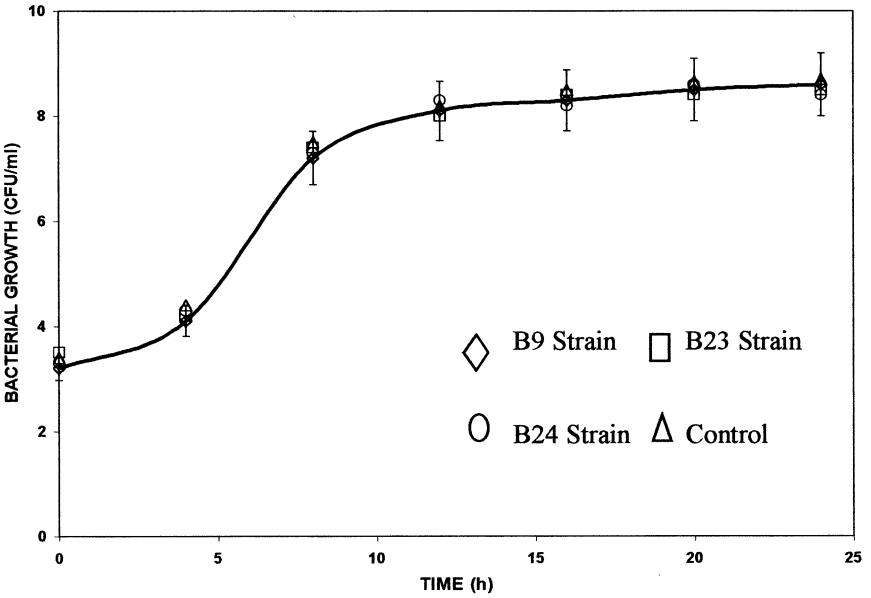

Figure 2. Bacterial stability tests. DmpR B9, B23 and B9 strains cultured in untreated pulp bleaching effluent for 24 h at 37°C

The effect of real industrial effluents on bacterial stability was assessed. Mutant DmpR B9, B23, and B24 strains were cultured in untreated pulp bleaching effluent for 24 h. Results summarised in Figure 2 show that all three mutant DmpR strains presented a stable growth over 24 h culture, under conditions where effluents were the sole carbon source. Moreover, bleaching effluents used here presented no inhibitory effect on bacterial growth.

The ability to detect chlorinated phenols in real effluents was assessed using two raw kraft cellulose bleaching effluents, containing 22 and 7 (mg/l) AOX, respectively. As seen in Table 2, all three strains showed enzymatic activity induction. However, the B24 strain presented responses consistent with the AOX concentration differences of the tested effluents, showing that this strain is better adapted to these type of effluents. This is a key issue, since real applications could be impaired if the effluent inhibited the activation of the enzymatic activity (Willardson et al. 1998).

The capacity of three mutant DmpR strains, B9, B23 and B24, to detect chlorinated phenolic compounds present in kraft pulp bleaching effluents was shown here. Whole-cell bacterial biosensors have the potential to provide inexpensive, easy-to-use methods of detecting industrial pollution.

Acknowledgments. This work was financially supported by FONDECYT N° 1010881, to whom the authors are thankful.

Table 1. Response of mutant DmpR strains to key organic pollutants.

	β galactosidase activity (min^{-1})		
Tested Compounds 100 μM single solutions	B9 strain	B23 strain	B24 strain
Blank	18 ±0.4	16 ± 0.2	20 ± 0.6
2-chlorophenol	198 ± 0.5	299 ± 0.8	301 ± 0.5
3-chlorophenol	180 ± 0.8	246 ±0.5	304 ± 4.6
4-chlorophenol	137 ± 0.5	319 ± 2.1	254 ± 0.9
2,4-dichlorophenol	122 ± 0.5	267 ±1.9	110 ± 0.5
2,4,6-triclorophenol	38 ± 0.4	51 ± 0.9	68 ± 0.8
2,4,5-triclorophenol	42 ± 0.3	64 ±0.8	75 ±1.3
3-chloro-4-methylphenol	66 ± 0.5	175 ± 2.1	99 ± 0.5
Phenol	186 ± 0.5	268 ± 2.1	314 ± 0.5
2,4-dimethylphenol	90 ± 1.0	295 ± 1.2	116 ± 0.8

β-galactosidase activity = $1000 \times A_{415} / \text{time} \times A_{595}$

Table 2. Response of mutant strains to kraft cellulose bleaching effluents.

		Mutant DmpR strains		
		B9	B23	B24
Bleaching Effluent	AOX (mg/l)	β galactosidase activity (min^{-1})		
Bleaching 1	22	74	53	104
Bleaching 2	7	44	44	68

β-galactosidase activity = $1000 \times A_{415} / \text{time} \times A_{595}$

REFERENCES

Aizawa M, Nishiquchi K, Imamura M, Kobatake E, Haruyama T, Ikariyama, Y (1995) Integrated molecular systems for biosensors. Sensors Actuators B24:1-5.

Applegate B, Kehrmeyer Y, Sayler G (1998) A chromosomally based *tod-luxCDABE* whole-cell reporter for benzene, toluene, ethylene, and xylene (BTEX) sensing. Appl Environ Microbiol 64:2730-2735.

Bale M, Fry J, Day M (1988) Transfer and occurrence of large mercury resistance plasmid in river epilithin. Appl Environ Microbiol 54: 972-978.

Burlage R, Palumbo A, Heitzer A, Sayler G (1994) Bioluminescent reporter bacteria detect contaminants in soil samples. Appl Biochem Biotechnol 45:731-740.

De Lorenzo V, Fernández S, Herrero M, Jakubzik U, Timmis N (1993) Engineering of alkyl- and haloaromatic-responsive gene expression with mini-transposons containing regulated promoters of biodegradative pathways of *Pseudomonas*. Gene 130:41-46.

Federal register (1998) National recommended water quality criteria. Fed Reg 63: 67547-67558.

Fleming BI (1995) Organochlorine in perspective. Tappi Journal 78:93-98

Harayama S, Timmis K (1992) Aerobic biodegradation of aromatic hydrocarbons by bacteria, p. 99-156. in H. Sigel and A. Sigel (ed), Metal ions in biological systems, Vol.28 Marcel Dekker Inc. New York, N.Y.

Heitzer A, Malachowsky K, Thonnard J, Bienkowski P, White D, Sayler G (1994) Optical biosensor for environmental on-line monitoring of naphthalene and salicylate bioavailability with an immobilized bioluminescent catabolic reporter bacterium. Appl Environ Microbiol 60:1487-1494.

Heitzer E, Webb O, Thonnard, J, Sayler G (1992) Specific and quantitative assessment of naphthalene and silicylate bioavailability by using a bioluminescent catabolic reporter bacterium. Appl Environ Microbiol 58:1839-1845.

Ikariyama Y, Nishiguchi S, Koyama T, Kobatake E, Aizawa M (1997) Fiber-optic based biomonitoring of benzene derivatives by recombinant *E. coli* bearing luciferase gene-fused TOL-plasmid immobilised on the fiber-optic end. Anal Chem 69:2600-2605.

King J, DiGrazia P, Appleagate B, Burlage R, Sanseverino J, Dunbar P, Larimer F, Sayler S (1991) Rapid, Sensitive bioluminescence reporter technology for naphthalene exposure and biodegradation. Science 249:778-781.

Martinez D, Pocurull E, Marce R, Borrul F, Calull M (1996) Separation of priority phenols by capillary zone electrophoresis with ultraviolet detection. J Chromatog 734:367-373.

Miller J (1972) A short course in bacterial genetics. Cold Spring Harbour laboratory Press. Cold Spring Harborr, N.Y.

Rasmussen L, Turner R, Barkay T (1997) Cell-density-dependent sensitivity of a mer-lux bioassay. Appl Environ Microbiol 63:3291-3293.

Scheller F, Shubert F (1992) Biosensors. Techniques and instrumentation in analytical chemistry, Vol. 11, p.359. Elsevier, Amsterdam.

Scheper T (1992) Biosensors for process monitoring. J Ind Microbiol 9:163-172

Shingler V, Moore T (1994) Sensing of aromatic compounds by the DmpR transcriptional activator of phenol-catabolizing *Pseudomonas* sp. strain CF600. J Bacteriol 176: 1555-1560.

Willardson B, Wilkins J, Rand T, Schupp J, Hill K, Keim P, Jackson P (1998) Development and testing of a bacterial biosensor for toluene-based environmental contaminants. Appl Environ Microbiol 64:1006-1012.

Wise A, Kuske C (2000) Generation of novel bacterial regulatory proteins that detects priority pollutant phenols. Appl Environ Microbiol 66:163-169.