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Marine Bacteria Tolerant to Chlorophenols

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The cellulose industry produce chlorophenols as a result of the use of chlorine during pulp cellulose bleaching, and are discharged into natural waters by their effluents. The pollution of coastal sea waters by chlorophenols and several other organic compounds could modify the biota of this environment because the compounds have high toxicity, are recalcitrant, and are accumulated in sediments or at different levels in the trophic chain (Fleming 1995). Despite the toxic properties of chlorophenols, many bacteria isolated from natural environments are tolerant to various concentrations of these chemicals, and some strains have demonstrated the ability to degrade these molecules (Saber and Crawford 1985). In addition, the enzymes involved in chlorophenol degradation and their genetic origin have been reported (Cork and Khalil 1995).

The Arauco Gulf posses a high biological productivity, despite its contamination with chlorophenols due to industrial effluents discharging. This environment has demonstrated stability to the impact of contaminants (Acuña and Chuecas 1994), and the basis for this behavior is still being studied. We investigated the effect of chlorophenol on bacterial viability using a population of aerobic heterotrophic bacteria isolated from coastal sea water near a discharging cellulose pulp mill. The aim of this work was to determine if chlorophenol tolerant bacteria or degradative bacteria may contribute to the stability of the Arauco Gulf ecosystem.

MATERIALS AND METHODS

Water samples were collected in the Arauco Gulf, central Chile, between 36°47' S and 37°10'S. This sector receives a bleached kraft mill effluent. The samples were obtained at 0.5 m of depth in two sites, one located 2,000 m away from the effluent discharge (site A), and the second sample at 50 m from the effluent discharge, on the plume of the effluent (site B). The water samples (1 L from each sector) were collected in May 1997 (autumn) using amber glass bottles and transported to the laboratory at 4°C. Oxygen was measured in situ with an oxygenmeter (Dissolved Oxygen Orion ATI), and pH and temperature were measured with a water quality checker (U-10 Horiba). Chlorinated chemicals

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were purchased from Aldrich Co., Milwaukee (>98% purity).

The effects of chlorophenol on the bacterial population of both sites were studied by adding 100 mL of each water sample to two 250 mL Erlenmeyer flasks. One flask of each sample was supplemented with 0.1 m*M* 2,4,6-trichlorophenol (2,4,6-TCP), and the second flask, without chlorophenol, was used as a control. The cultures were incubated for 5 d at 25°C with constant shaking (150 rpm), and total viable bacterial counts, chlorophenol tolerant bacterial counts, and 2,4,6-TCP concentration were measured each 24 hr during 5 d. Total viable bacterial counts were determined by placing, in triplicate, 20 µL of a ten-fold serial dilutions in saline solution (Saber and Crawford 1985) of each sample on the surface of R2A agar plates plus 1.5% of NaCl (Herbert 1990). The agar plates were incubated at 25°C for a further 5 d, and then bacterial counts were done. The counts of tolerant bacteria were determined as above in media supplemented with 0.1 m*M* 2,4,6-TCP, 2,4,5-trichlorophenol (2,4,5-TCP) or 4-chloro-3-metilphenol (4C-3MP). These compounds were chosen because they are frequently detected in this type of effluent (Kringtad and Lindström 1984). Concentration of 2,4,6-TCP was determined spectrophotometrically in supernatants obtained by centrifuging aliquots of 1.5 mL at 15,000g during 2 min. Aromatic ring cleavage was followed by spectral changes in the range of 200 nm to 350 nm (Cecil spectrophotometer, model 3,000).

To characterize some bacterial strains isolated from both sites, water samples were immediately inoculated on R2A agar plates free of chlorophenol, and incubated at 25°C for 5 d. Thirty colonies with different morphological aspects were selected from each sample and were characterized by Gram stain. Gram negative bacilli were further characterized on glucose-H_2S medium (Ward et al. 1986). All the strains were assayed for their tolerance to chlorophenols by using an agar dilution technique, as described by the National Committee (1990). Briefly, 1 x 10^6 ufc/mL were inoculated on R2A agar plates supplemented with 0.05, 0.25, 0.50, 1.00, or 2.00 m*M* of 2,4-diclorophenol (DCP), 2,4,5-TCP, 2,4,6-TCP, 4C-3MP or pentachlorophenol (PCP), respectively, using a Steer replicating apparatus (Steer et al. 1959), and the plates were incubated for 48 hr at 25°C. The tolerance level was defined as the highest concentration of chlorophenol allowing the growth of the bacterial strain under assay.

RESULTS AND DISCUSSION

The physical and chemical parameters of the sampling sites are shown in Table 1. There are differences in temperature, oxygen and salinity in both sectors, probably due to the effluent discharges. Higher temperature and lower pH of water were found near site B (discharging site).

The bacteriological studies also showed differences in the bacterial counts obtained in both sites. Total viable bacterial counts near the effluent were higher as compared with bacterial counts from site A (Figure 1, A and B). This is probably due to the presence of utilizable organic matter incorporated by the

Table 1. Physical and chemical parameters in the sample sites

Site	T ° C	pH	O_2 (mg l^{-1})	S (g l^{-1})
A	9	7.9	10.5	31
B	15	7.2	8.6	25

T= temperature centigrade; O_2= dissolved oxygen; S== salinity

cellulose effluent, that can be used as a carbon source by the bacterial population. In addition, a similar ratio of tolerant/non-tolerant bacterial counts were found along the incubation period (Figure 1, A and B). This result suggest that in both sites there exists a fraction of the bacterial population which is intrinsically tolerant to 2,4,6-TCP, but neither 2,4,5-TCP nor 4C-3MP tolerant bacteria were recovered. On the other hand, the addition of 2,4,6-TCP to the samples allowed the recovery of bacteria tolerant to 2,4,5-TCP, indicating that 2,4,6-TCP also selected for 2,4,5-TCP tolerant bacteria. Probably bacteria tolerant to both compounds are present in a small fraction in the environment (Figure 1, C and D). Furthermore, the increase in 2,4,5-TCP tolerant bacterial counts were higher in site A than in site B (Figure 1, C and D), suggesting that environments less exposed to the contaminants, allow the existence of more genetic and physiological diversity among the bacterial population in agreement with Dean-Ross and Mill (1989).

To assess whether the bacterial strains isolated from a polluted and non polluted environment showed differences in their growth rate when challenged with chlorophenol compounds, 2,4,6-TCP was added to the water samples obtained from sites A and B prior to searching for tolerance to chlorophenols. The results indicate a rapid response of the bacterial population from site B (contaminated) to the pollutant, since both the tolerant bacterial count and the total viable count became equal in less than one day. On the other hand, the response observed with the bacterial population from site A (non contaminated) was delayed as compared with site B (Figure 1, C and D), probably due to either a longer adaptation period of tolerant bacteria or because of their small number in the samples.

It is interesting to point out that during the experimental work, the total viable counts in water samples from site A (Figure 1, C) were higher in the presence of 2,4,6-TCP as compared with the control without chlorophenol (Figure 1, A). This result may be an indirect effect of 2,4,6-TCP, since the compound could be toxic for protozoa that predate bacteria, allowing a faster increase in bacterial number.

Degradation of 2,4,6-TCP, measured spectrophotometrically after 5 d of incubation, was 15% and 35% in the samples from sites A and B, respectively (data not shown). This behavior may be explained if we consider that the adaptation of bacteria to various environmental trophic conditions could be

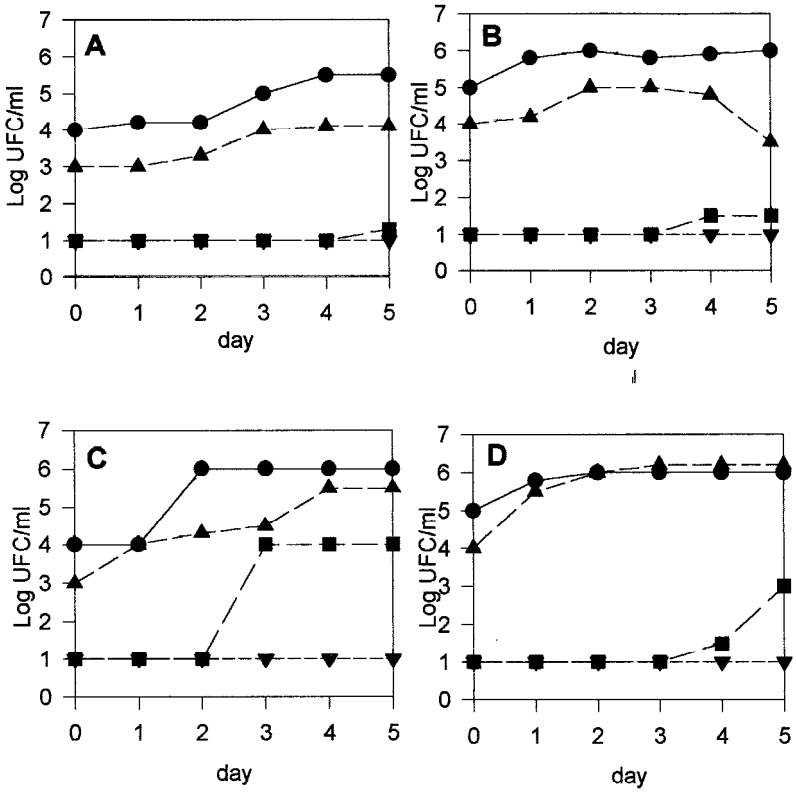

Figure 1. Viable bacterial counts. Panel A, site A; Panel B, site B; Panel C, site A plus 0.1mM 2,4,6-TCP; Panel D, site B plus 0.1mM 2,4,6-TCP. Symbols indicate total viable bacterial counts (●), tolerant bacterial counts to 2,4,6-TCP (▲), 2,4,5-TCP (■), and 4C-3MP (▼).

Table 2. Percentage of nonfermentative Gram negative bacilli tolerant to Chlorophenol in seawater samples from Arauco gulf

Site	Chlorophenol	Number of Strain	Chlorophenol Concentration (m*M*)				
			0.05	0.25	0.50	1.00	2.00
A	4C-3MP	30	100	40	12	0	0
	DCP	30	100	60	20	0	0
	2,4,5-TCP	30	2	0	0	0	0
	2,4,6-TCP	30	100	100	100	90	70
	PCP	30	40	25	0	0	0
B	4C-3MP	30	100	60	40	30	0
	DCP	30	100	100	100	100	90
	2,4,5-TCP	30	15	0	0	0	0
	2,4,6-TCP	30	100	100	80	80	80
	PCP	30	40	30	0	0	0

4C-3MP= 4-chloro-3-metilphenol; DCP= 2,4-dichlorophenol; 2,4,5-TCP= 2,4,5- trichlorophenol; 2,4,6-TCP= 2,4,6-trichlorophenol

influenced by the presence of more than one kind of organic matter as was proposed by Kuiper and Hanstveti (1984). The authors suggest that any rapid degradable organic substrate in seawaters may delay the degradation of chlorophenol by bacteria.

We selected 15 strains in plates inoculated with samples from site A and 15 strain from site B to further study their tolerance level to five chlorophenols (Table 2). All the strains isolated were characterised as Gram negative nonfermentative bacilli. The tolerance test of this bacterial strains showed that 2,4,6-TCP was the most tolerated chlorophenol and 2,4,5-TCP, the less tolerated. The tolerance to these compounds was related to the position of the chlorine in the aromatic ring, rather than to the number of chlorine in the molecule, since 2,4,5-TCP and 2,4,6-TCP showed differences in their tolerance levels. Also DCP was tolerated better than 2,4,5-TCP but was less tolerated than 2,4,6-TCP. The results are similar to those reported by Ruckdeschell et al. (1987) with several bacterial species, and agreed with our results obtained with bacteria isolated from freshwater (Godoy et al. 1998). The authors also found a strong correlation between the antibacterial activity of chlorophenol and the position of chlorine in the aromatic ring.

In general, chlorophenol tolerance observed among bacterial strains isolated from marine environments could be the consequence of a selective pressure exerted by halogenated organic molecules naturally synthesised by marine organisms like *Rhodophytas poriferas* or nemerteas in pristine seawater (Gary 1988). Also, genes that encode for enzymes able to degrade a variety of aromatic compounds has been described in a marine bacterium (Wang et al. 1996). The results presented demonstrate that in the Arauco Gulf seawater there are aerobic heterotrophic bacteria able to survive up to 0.1 m*M* (20 ppm) of 2,4,6-TCP, while contaminated environment contains close to 10 ppb of chlorophenols (Wegman et al. 1979). This is important to keep in mind because a high proportion of natural bacteria present in contaminated environments are tolerant to the toxicity of the chlorophenol, remain viable, and some of them are able to degrade these compounds. Therefore, in marine environment contaminated with chlorophenol, native bacteria populations, might be involved in natural physiological processes tending to recover the pristine condition of the ecosystem.

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