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Toxicity of Organotins Towards the Marine Yeast *Debaryomyces hansenii*

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Abstract. Of nine organotin compounds tested towards the marine yeast *Debaryomyces hansenii*, only triphenyltin chloride (Ph_3SnCl) and mono-, di-, and tributyltin chloride induced significant K^+ release from cells which was symptomatic of viability loss. The general order of toxicity of the butylated compounds was tributyltin chloride (Bu_3SnCl) > monobutyltin chloride ($BuSnCl_3$) ≫ dibutyltin chloride (Bu_2SnCl_2). The overall toxicity of Ph_3SnCl was similar to $BuSnCl_3$. Release of K^+ induced by butylated tin compounds or by Ph_3SnCl was strongly dependent on the external pH. Maximal toxicity occurred at pH 6.5 for Bu_3SnCl, $BuSnCl_3$, and Ph_3SnCl, whereas maximal toxicity of Bu_2SnCl_2 occurred at pH 5.0. Toxicity was decreased above or below these values. The toxicity of $BuSnCl_3$, Bu_3SnCl, and Ph_3SnCl was reduced at salinity levels approximating to sea water conditions. Prior growth of *D. hansenii* in 3% (w/v) NaCl also resulted in reduced sensitivity to Bu_3SnCl as evidenced by a decreased rate and extent of K^+ efflux. Bu_3SnCl-induced Na^+ release from cells grown in the absence or presence of 3% (w/v) NaCl was low and similar in both cases. It appeared that the monovalent cation was important in the reduction of Bu_3SnCl toxicity since Na_2SO_4 had a similar protective effect as NaCl while CsCl completely prevented K^+ efflux. Thus, the effects of external NaCl were related both to Na^+ and to Cl^-. These results emphasize that cellular and environmental factors influence the toxic effects of organotins and suggests that these compounds may be more effective antimicrobial agents in some environmental niches than in others.

Introduction

In the series R_nSnX_{4-n} (where n = 0 − 4 and X = an anion), mono- ($RSnX_3$) and disubstituted (R_2SnX_2) organotins are used as stabilizers in polyvinylchloride beverage bottles, food packaging materials, and water distribution pipes, as catalysts in several industrial processes, as coatings in glass, as additives in poultry feeds to control infectious agents, and for other purposes. Trisubstituted (R_3SnX) organotins are used mainly as antibiologic agents [41] while tetrasub-

stituted (R_4Sn) organotins are used mainly for the synthesis of organotins from $SnCl_4$ [5, 14, 43 and Cooney and Wuertz, unpublished data]. Recently, attention has been focused on tributyltin, an effective component of antifouling paints, which is possibly the most toxic compound man has ever introduced to the aquatic environment [8, 32, 44]. Tributyltins can be degraded under environmental conditions, and the principle factors limiting degradation appear to be photolysis in water and biologic degradation in water and sediment [28, 33]. Methyltins can be formed under environmental conditions aerobically [9, 12, 23, 24, 27, 29] and anaerobically [22] by microorganisms as well as by abiotic mechanisms [10, 11, 15, 43].

In general, organotin toxicity to microorganisms decreases in the order $R_3SnX > R_2SnX_2 > RSnX_3$, while R_4Sn compounds have little toxicity. The nature of the anion, X, has little effect on toxicity. Although there are species and strain variations, triorganotins with ethyl, propyl, butyl, pentyl, or phenyl R groups are generally toxic to microorganisms, with tripropyl-, tributyl-, and triphenyltins usually being the most toxic (Cooney and Wuertz, unpublished data) and [31]. The toxicity of organotins in aqueous systems is related to total surface area of the molecule (TSA) and to the compound's octanol: water partition coefficient (K_{ow}). Thus, it has been suggested that organotins become associated with the surfaces of biologic membranes rather than penetrating them [7, 16]. Consequently, toxic effects of organotins should first be manifest at the cell membrane, and the estimation of K^+ release can provide a rapid measure of toxicity as previously described for other heavy metal compounds, certain antibiotics, and xenobiotics [4, 17, 18, 42, 46, 47].

Yeasts provide an easily manipulated eukaryotic cell system, and various species of environmental (and medical) significance are receiving increasing use for toxicity testing of antibiologic compounds [13, 17, 21, 42, 47]. In an earlier work, we showed that a number of yeasts, including several isolated from marine ecosystems, were sensitive to organotins. Of the organotins examined, tributyltin (Bu_3SnCl) was the most toxic, followed by monobutyl- ($BuSnCl_3$), triphenyl- (Ph_3SnCl), and dibutyltins (Bu_2SnCl_2). Trimethyl-, triethyl-, diethyl-, dimethyl-, and monomethyltins were less toxic or had no effect. Among four methods used to screen the organotin compounds, the release of K^+ from cell suspensions, measured using a K^+-electrode, provided quantitative information and allowed for comparisons between organisms and among compounds [13].

Preliminary work indicated that K^+ efflux from *Zygosaccharomyces rouxii* was affected by the presence of NaCl [13]. This suggested that the toxicity of organotins might vary among fresh, estuarine, and marine waters. The present work was undertaken in order to expand these observations and obtain additional information about organotin-induced K^+ release, including the influence of external pH and salinity, using as a test organism a marine isolate of *Debaryomyces hansenii*. This yeast is widely distributed in saline aquatic environments, and a considerable amount of work on the physiology of osmotolerance has been carried out [40]. In addition, the greater tolerance of *D. hansenii*, as compared with bacteria, towards stannic chloride, methyl- and most ethyltin compounds has been documented previously [12, 26].

Methods

Organism, Media and Growth Conditions

A marine isolate of *Debaryomyces hansenii* (NS-75-21), kindly provided by Dr. S. A. Crow, Department of Biology, Georgia State University, Atlanta, Georgia, was maintained on MYGP agar of composition (gl^{-1}): malt extract (Lab M), 3; yeast extract (Lab M), 3; D-glucose, 10; bacteriologic peptone (Oxoid), 5; agar (Lab M, No. 2), 12. Experimental cultures were grown at 25°C on an orbital shaker (100 rpm) in a medium comprising (gl^{-1}): KH_2PO_4, 2.72; $K_2HPO_4.3H_2O$, 5.22; $(NH_4)_2SO_4$, 2.0; $MgSO_4.7H_2O$, 0.12; $FeSO_4.7H_2O$, 0.0022; $ZnSO_4.7H_2O$, 0.004; $MnSO_4.4H_2O$, 0.004; $CuSO_4.5H_2O$, 0.0004; D-glucose, 20.0; yeast extract (Difco), 1.0. Where appropriate, NaCl was added to a final concentration of 30 gl^{-1}. Cells were counted using a modified Fuchs-Rosenthal hemocytometer after appropriate dilution with distilled water. Viability was estimated by the conventional spread-plate method on MYGP agar using sterile distilled water as diluent.

Nine organotin compounds were used. CH_3SnCl_3, $(CH_3)_2SnCl_2$, $(CH_3)_3SnCl$, and mono-, di-, and tri-*n*-butyltin chlorides were from Alfa Products, Danvers, MA. $(C_2H_5)_2SnCl_2$ and $(C_2H_5)_3SnBr$ were from the Aldrich Chemical Company, Milwaukee, WI. Triphenyltin chloride was from Fluka AG, Buchs, Switzerland. Stock solutions of these compounds were prepared in absolute ethanol.

Potassium Release

Exponentially growing cells were harvested by centrifugation (5 min, 1,200 × *g*), washed three times with and finally resuspended in 5 mM piperazine-N,N′-bis [2-ethanesulphonic acid] (PIPES) buffer, adjusted to pH 6.5, unless stated otherwise, using solid tetramethylammonium hydroxide, to a cell density of approximately 2×10^8 ml^{-1}. For experiments, cell suspensions were derived from this by dilution in PIPES buffer to a cell density of 10^7 ml^{-1} and contained D-glucose at a final concentration of 50 mM. After equilibration at 25°C on a magnetic stirrer for 10–15 min, organotin compounds were added from stock solutions to a final concentration of 50 μM unless stated otherwise. K^+ release was measured using a K^+-specific electrode (Russell pH Ltd, Auchtermuchty, Fife, Scotland) connected to a KENT/EIL pH/ion meter, model 7055, and a Servoscribe potentiometric recorder for continuous measurements [21, 46, 47]. Where appropriate, aliquots of NaCl, Na_2SO_4, CsCl, or $CaCl_2.2H_2O$ solutions were added 15 min before the addition of tributyltin. Where applicable, Na^+ and K^+ concentrations in supernatant buffer or acid digests of cell pellets were determined using a Pye Unicam SP900 atomic absorption spectrophotometer, as previously described [17, 18].

Results and Discussion

The toxicity of nine organotin compounds towards *D. hansenii,* as assessed by K^+ release, is shown in Figure 1. The greatest loss of K^+ was induced by Bu_3SnCl followed by Ph_3SnCl and $BuSnCl_3$. Maximal rates of K^+ release for Bu_3SnCl, Ph_3SnCl, and $BuSnCl_3$ were 2.58, 2.15, and 2.27 nmol K^+ min^{-1} $(10^7$ cells$)^{-1}$, respectively (data calculated from Fig. 1). Bu_2SnCl_2 resulted in a lower extent of K^+ efflux (rate = 0.21 nmol K^+ min^{-1} $(10^7$ cells$)^{-1}$), but $MeSnCl_3$, Me_2SnCl_2, Me_3SnCl_3, Et_3SnBr, and Et_2SnCl_2 showed little or no effect (Fig. 1).

K^+ is actively concentrated in fungal cells, uptake being coupled to the plasma membrane ATPase-dependent H^+ efflux via the membrane potential [6, 36, 39]. K^+ efflux from cells frequently accompanies heavy metal uptake by fungi,

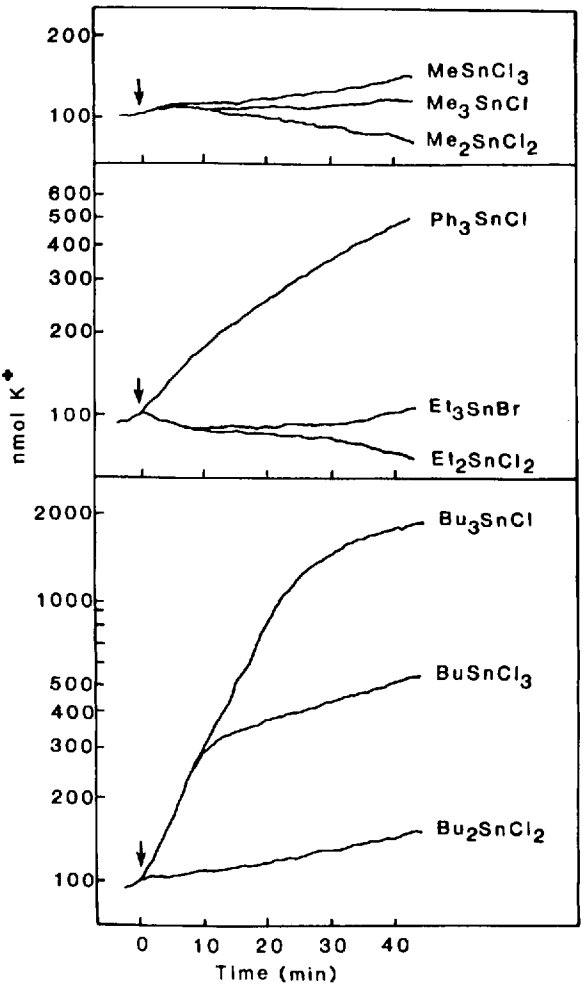

Fig. 1. K^+ release from *D. hansenii* induced by organotin compounds. The addition of organotin to the yeast suspension is indicated by the arrow. The final concentration of organotins was 50 μM except for Et_3SnBr and Et_2SnCl_2 where the concentration was 5 μM due to increasing insolubility above this concentration. Typical traces are shown from one of three experiments.

though not in all cases. When toxic effects are manifest or the cell populations exhibit mixed sensitivities, K^+ release may show no simple stoichiometry with metal uptake [4, 18, 19, 30, 34, 35, 42]. Nevertheless, in many instances K^+ efflux provides a reasonable measure of toxicity, probably reflecting damage to cell membranes [17, 18, 35], and enables comparison among different species and strains and different toxic compounds [13, 17, 46, 47]. In this study, the connection between toxicity and K^+ release from *D. hansenii* was clear and after 30 min exposure to $BuSnCl_3$, Bu_3SnCl, or Ph_3SnCl, plate counts on MYGP medium showed a >99% kill whereas there was negligible viability loss with Bu_2SnCl_2 (results not shown).

A marine isolate of the fungus *Aureobasidium pullulans* showed similar sensitivity to the three butyltins but was not as sensitive to Ph_3SnCl. The yeasts *Pichia pinus, Saccharomyces cerevisiae,* and *Zygosaccharomyces rouxii,* showed individual patterns of sensitivity to these four compounds [13]. It was initially

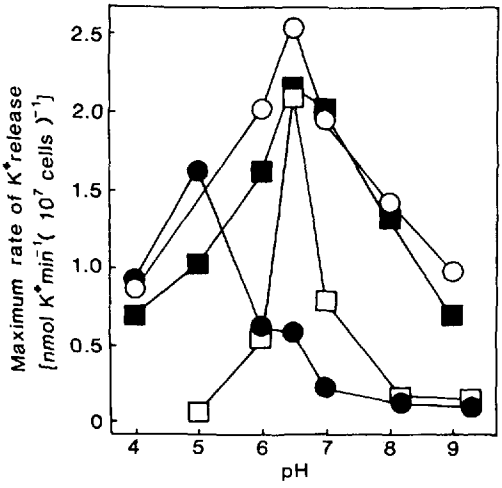

Fig. 2. Effect of external pH on organotin-induced K^+ release from *D. hansenii*. (○) Bu_3SnCl; (●) Bu_2SnCl_2; (□) $BuSnCl_3$; (■) Ph_3SnCl. Final concentration 50 μM. Maximal rates of K^+ release calculated from traces similar to those shown in Figure 1; typical results are shown.

surprising that $BuSnCl_3$ was more toxic than Bu_2SnCl_2 in this and a previous study [13] because disubstituted organotins are generally regarded as stronger antimicrobial agents than monosubstituted organotins [26, 37, 38]. Strain specificity appears to be important because a strain of *D. hansenii* isolated from Chesapeake Bay was sensitive to R_3Sn compounds including Et_3-, Pr_3-, Bu_3-, and Ph_3Sn compounds, but not to $BuSnCl_3$ [26].

K^+ release from *D. hansenii* induced by butyltins and Ph_3SnCl was strongly dependent on the external pH (Fig. 2). Maximal toxicity occurred at pH 6.5 for $BuSnCl_3$, Bu_3SnCl, and Ph_3SnCl while maximal toxicity of Bu_2SnCl_2 occurred at pH 5.0. This explains the toxicity sequence $Bu_3SnCl > BuSnCl_3 > Bu_2SnCl_2$ mentioned above. In a screening experiment that employed two-dimensional gradient diffusion plates, the toxicity of Bu_3SnCl to *A. pullulans* was dependent on external pH, and toxicity decreased above pH 5.5 [13]. Thus, there is clear evidence that pH can influence the toxicity of organotins. The differences in toxicity between Bu_3SnCl and Bu_2SnCl_2 may be related to the fact that trisubstituted organotins appear to affect membranes while disubstituted organotins react with dithiol groups on enzymes and coenzymes (Cooney and Wuertz, unpublished data) and [15]. External pH may influence the interaction of all surfaces with organotins.

Both the initial rate and final amount of K^+ released after exposure of *D. hansenii* to Bu_3SnCl, $BuSnCl_3$, or Ph_3SnCl was reduced in the presence of 1.5 or 3% (w/v) NaCl, approximating to estuarine and marine conditions, respectively (Fig. 3). Cells of *D. hansenii*, which had been previously grown in 3% (w/v) NaCl, also released less K^+ when exposed to Bu_3SnCl than did cells grown in unsupplemented medium (Fig. 4). Bu_3SnCl induced a low release of Na^+ from cells pregrown in unsupplemented or salt-amended media which was similar in both cases (Fig. 4). This indicated that the observed depression of tributyltin-induced K^+ efflux by NaCl was a genuine response and not due to the increased replacement of cellular K^+ by Na^+. Intracellular concentrations of Na^+ in cells grown in the absence or presence of 3% (w/v) NaCl were 1.9 ±

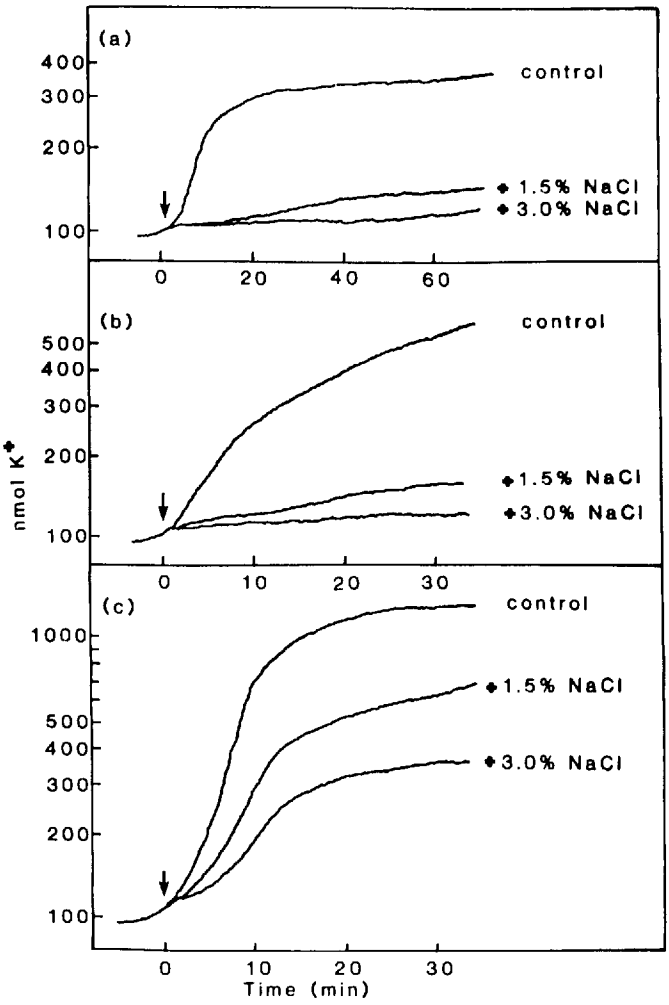

Fig. 3. Effect of external NaCl on organotin-induced K^+ release from *D. hansenii*. **(a)** $BuSnCl_3$; **(b)** Ph_3SnCl; **(c)** Bu_3SnCl. The final organotin concentration was 50 μM. Addition of organotin to the yeast suspension is indicated by the arrow. Typical results are shown from one of three experiments.

0.3 and 33.7 ± 1.3 mM, respectively (corresponding concentrations of cellular K^+ were 284.0 ± 11.0 and 92.5 ± 2.8 mM). It has been documented that with repeated culture of yeast, there can be partial or complete replacement of cell K^+ with other cations, e.g., Na^+, Ca^{2+}, and Mg^{2+} [6], and when Na^+-loaded *S. cerevisiae* were exposed to heavy metals, Na^+ was released instead of K^+ [35]. However, although there was some alteration in relative K^+ and Na^+ concentrations inside cells, this did not occur in this study. We have previously shown that in exponentially growing *D. hansenii,* monovalent cations make only a minor contribution to internal osmotica, and organic solutes, mainly glycerol, have the major role in contributing to internal osmolality [40]. It should be stated further that internal accumulation of glycerol may contribute to the increased tolerance shown by salt-grown *D. hansenii* to tributyltin since elevated

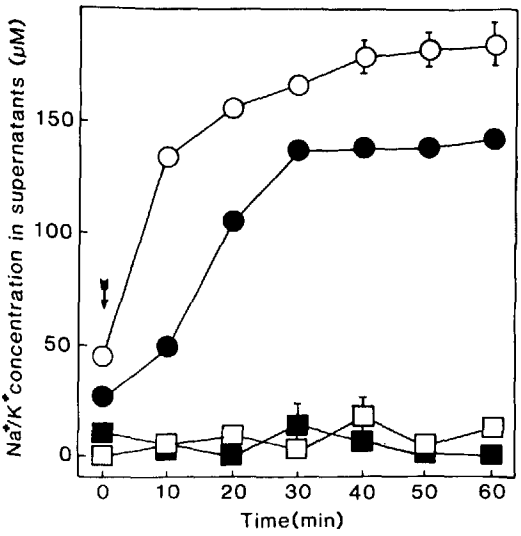

Fig. 4. Effect of Bu_3SnCl on K^+ and Na^+ release from *D. hansenii* grown in the absence or presence of NaCl. (○ ●) K^+; (□ ■) Na^+. Open symbols refer to control medium, closed symbols to medium containing 3% (w/v) (=0.51 M) NaCl. Addition of Bu_3SnCl to a final concentration of 50 μM is indicated by the arrow. Average values shown; standard errors are indicated where possible.

internal osmolality may prevent influx of the toxic moiety. This has been shown for *Penicillium ochro-chloron* which synthesized elevated levels of intracellular glycerol in response to high external concentrations of $CuSO_4$ or NaCl, which appeared to contribute to the exclusion of copper from the cells [20]. Na_2SO_4 also reduced the extent of Bu_3SnCl-induced K^+ efflux in a manner similar to NaCl, suggesting that the Na moiety is important. $CaCl_2$ had a more pronounced protective effect, with significant K^+ release occurring only after a lag period of approximately 20 min whereas CsCl completely prevented K^+ release (Fig. 5), which may imply that the chloride ion is also important. Na_2SO_4 also depressed Ph_3SnCl-induced K^+ release (results not shown).

It has been suggested that triorganotins can act against fungi by interfering with mitochondrial function. Interaction with mitochondrial membranes can result in (1) large-scale swelling of the organelle; (2) discharge of an energy-dependent hydroxyl-anion gradient leading to limited swelling of membranes, due to the triorganotin compound acting as an ionophore; and (3) interference with the energy transduction apparatus [1, 2] and (Cooney and Wuertz, unpublished data). Dialkyltins cause similar effects on mitochondria but appear to act by binding to dithiol groups on enzymes and coenzymes [1, 3]. It has been proposed that trisubstituted organotins become associated with the surfaces of biologic membranes rather than penetrating them [7, 16]. It is difficult to envisage how organotins could affect mitochondria in yeast without penetrating and adversely affecting the cytoplasmic membrane. It is possible that in salt-grown cells, the NaCl leads to changes in cell membranes so that interactions with organotins are altered. There is precedent for this in that the transfer of the osmotolerant yeast *Zygosaccharomyces rouxii* from NaCl-free medium to one containing 2 M NaCl can alter membrane lipid composition [45]. In addition, it was proposed that a Bu_3Sn-resistant mutant of the bacterium

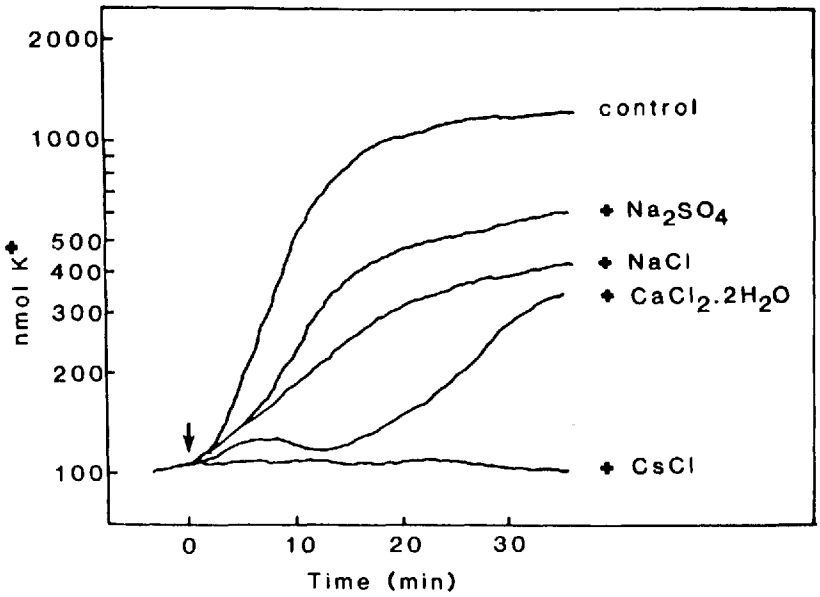

Fig. 5. The effect of various salts on Bu_3SnCl-induced K^+ release from *D. hansenii*. The final concentrations of the salts used were 3% (w/v) (=0.51 M) NaCl; 3% (w/v) (=0.18 M) CsCl; 1.5% (w/v) (=0.11 M) Na_2SO_4; 1.5% (w/v) (=0.10 M) $CaCl_2.2H_2O$. The addition of Bu_3SnCl to the yeast suspension, to a final concentration of 50 μM, is indicated by the arrow. Typical results are shown.

Bacillus subtilis owed its resistance to alteration in membrane phospholipids; the membrane lipids of the resistant mutant contained less monounsaturated C_{15} fatty acids [24]. The possible contribution of elevated internal osmolality, due to the internal synthesis of carbohydrates, e.g., glycerol, should not be discounted in salt-grown cells of *D. hansenii*, as mentioned above [20, 40]. Furthermore, external Na^+ may alter organotin toxicity by interfering with binding sites on the membrane and/or altering the chemical behavior of the organotin moiety, and such processes, which must include binding and accumulation of the organotins, appear to be strongly dependent on external pH.

It should be mentioned that the concentrations of organotin compounds used in this work may exceed levels encountered in the natural environment except at highly polluted sites. Another factor that should be borne in mind is that high concentrations of cells are needed to detect significant K^+ efflux [4, 17, 21] and there may be a cell number/[toxicant] relationship, particularly when binding is a prerequisite for toxic symptoms. Nevertheless, organotin concentrations found in the environment can cause significant impairment of yeast growth [13] and (unpublished data). It is likely that the effects of salinity and pH on organotin toxicity reported here may be even more significant at lower organotin concentrations. It is clear from this work that the responses of *D. hansenii* to organotins may depend on cell-mediated processes and environmental factors. Such effects may mean that organotins are more efficient antimicrobial agents in some environmental niches than in others.

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