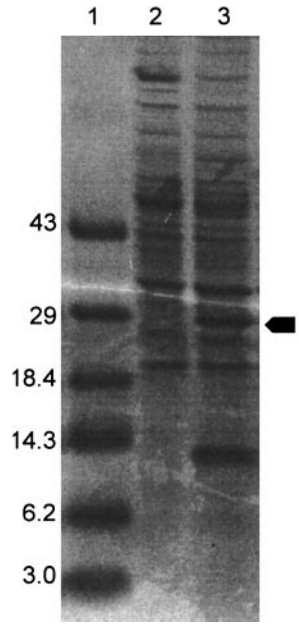

FIG. 3. SDS–PAGE gel showing separated denatured proteins from untransformed *B. subtilis* (lane 2) and from *B. subtilis* transformed with pUBxynA (lane 3). The correct size of the xylanase protein, approximately 29 kDa is indicated. Lane 1 shows the marker standards indicated in kDa.

colonies but not from pUB110-transformed colonies (Fig. 3). This is consistent with correct expression of the *xynA* gene contained in pUBxynA.

This is the first report of the transformation of *B. subtilis* using particle bombardment of submicrometer gold particles. The technique offers a means for testing transformation of recalcitrant bacteria when transformation has not been possible through other means. Compared to electroporation, the efficiency of transformation using ballistic technology with 0.3-μm gold particles is still low. However, when a high transformation efficiency is not required, as long as transformed bacterial cells are obtained, the method presents a promising alternative. Because the polyol-manufacturing process is capable of producing even smaller particles, of various elemental compositions, the potential for transformation of even smaller prokaryotes may be possible, and may lead to the transformation of species which are resistant to current transformation procedures.

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A Bioanalytical Method for the Monitoring of Metal Alkyls in Solution

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The monitoring of many metal alkyls in solution is a very important problem of environmental concern, since many of these compounds are toxic. For example, triethyl lead [$(Et)_3$ Pb-Cl] is a neurotoxic compound which is produced by biodegradation of tetraethyl lead [$(Et)_4$ Pb], largely used as antiknock compounds in fuels (1–4). Analogously trialkyltin compounds have been largely used as aquatic antifoulant biocides for boating and aquacultural industries (5). Metal alkyls are lipophilic; they are bioaccumulated by the aquatic food chain and are toxic to aquatic biota at concentrations as low as 10 μg/liter (6). Recent legislation restriction for the use of

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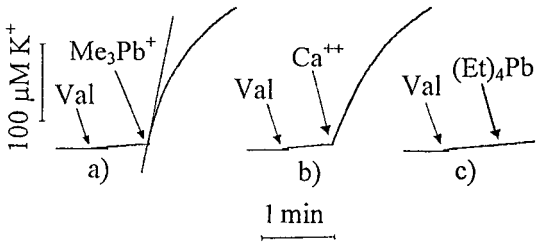

FIG. 1. A typical experiment of release of K^+ by nonrespiring mitochondria (1 mg/ml) in the presence of a permeant cation. In (a) the permeant lipophilic cation is $Pb(Me)_3^+$ (200 μM). In (b) it is Ca^{2+} (150 μM). In (c) no significant potassium release occurs when $(Et)_4$ Pb (100 μM) is added since this compound does not form a cation in solution. In (a), the dotted line is the initial rate of potassium efflux. Medium composition: 0.25 M sucrose, 10 mM Mops, pH 7.4, 0.1 mM EGTA, 40 μM KCl. In all experiments were added 2 nmols/mg protein of valinomycin (Val).

trialkyltin compounds in aquatic systems has reduced the scope of environmental contamination. However, some harbor estuaries remain exposed because large vessels still use tributyltin-based antifoulant paints.

Analogously methyl mercury [Me-Hg-Cl], a lipophilic mercurial neurotoxicant, is one of the agents that has caused severe human poisonings of environmental origin (7–9).

In this paper we propose a simple method to detect the presence of such compounds in solution. The method utilizes the mitochondria as biosensors and allows the monitoring of all organometals which are able to form a lipophilic cation in solution.

The responses of the proposed method are similar to the "red killifish" toxicological test. The procedure, therefore, can be utilized to predict the response of that toxicological test (OECD test guideline).

Materials and methods. Rat liver mitochondria were prepared following the usual procedures (10); the protein concentration was determined by the Lowry method (11).

The potassium concentration in the medium was monitored (Radiometer Copenhagen PHM 84 recording apparatus Linseis) by a potassium selective electrode (Ingold). The reaction vessel containing the medium (4 ml) was stirred at 25°C.

Medium composition was 0.25 M sucrose, 10 mM Mops, pH 7.4, 0.1 mM EGTA, 40 μM KCl, 0.5 mg/ml mitochondria. Valinomycin (2 nmol/mg of protein) was added before the alkyl metal compound. The addition of mitochondria to the medium (0.5 mg/ml) implies a further addition of K^+ arising from the mitochondrial content. Since the uptake rate is potentially ($\Delta\Psi$) dependent on an exponential correlation (12), to ensure the same driving force ($\Delta\Psi$) in all experiments, the external K^+ concentration was adjusted to 60 μM by addition of K^+. Under this condition the $\Delta\Psi$, calculated by means of the Nernst formula $\Delta\Psi = RT/F \, \mathrm{Log}[K^+]_{in}/[K^+]_{out}$, is 194 mV.

Valinomycin (Sigma, Milan) and all reagents were of analytical grade. Organometal compounds Bu_3-Sn-Cl, Bu_2-Sn-Cl_2, Bu-Sn-Cl_3, Phe-Sn-Cl_3, Me_3-Sn-Cl, Me_2-Sn-Cl_2, Me-Hg-Cl, and Et-Hg-Cl were purchased from Sigma–Aldrich "Inorganics & Organometallics." Pure Et_3-Sn-Cl, Pro_3-Sn-Cl, Phe_3-Sn-Cl, and Phe_2-Sn-Cl_2 compounds, prepared by redistribution reactions (13), were obtained by recrystalization from petroleum ether (40–60°C). Pure cyclohexyl$_3$-Sn-Cl was obtained following the synthesis procedure described by Krause and Pohland (14). Pure Me_3-Pb-Cl and Bu_3-Pb-Cl were prepared following the standard procedures (15, 16) with further chromatografic purification on silica gel column (Kieselgel 60 (70–230 mesh)) using petroleum ether as eluent.

The choice of $v = 300$ nmol/mg/min of K^+ as the reference rate to compare the uptake rate (and therefore the sensitivity) of the tested compounds was suggested because this rate is reached by all the tested compounds; note that rate values higher than 300 nmol/mg/min are not reached by all the examined compounds and not all the compounds are soluble enough to reach high transport rates. Moreover, some compounds at high concentrations interfere with the electrode.

The EC_{300} standard deviation values (i.e., the concentrations of toxic compounds which give rise to a rate of 300 nmol K^+/mg of protein/min) were calculated for each compound from the standard deviation of the slope of the straight line V_{K+} vs concentration by means of the computer program Microcal ORIGIN 4.1 for Window 95.

Results and discussion. In aqueous solution organometal compounds of the general formula $(alkyl)_m$-Me-X_n undergoes dissociation, giving rise to the following reactions (17–20):

$$(alkyl)_n\text{-Me-}X_m \Leftrightarrow (alkyl)_n\text{-Me-}X^+_{(m-1)} + X^-$$

$$(alkyl)_n\text{-Me-}X^+_{(m-1)} \Leftrightarrow (alkyl)_n\text{-Me-}X^{2+}_{(m-2)} + X^-$$

$$(alkyl)_n\text{-Me-}X^{2+}_{(m-2)} \Leftrightarrow (alkyl)_n\text{-Me-}X^{3+}_{(m-3)} + X^-$$

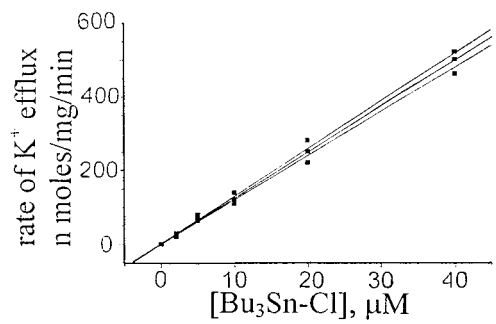

FIG. 2. Dependence between the initial rate of potassium efflux and $(Bu)_3$Sn-Cl concentration. Values are the average of three experiments. Medium composition and experimental conditions are as indicated under Materials and methods.

The presence in solution of a lipophilic cation (which is also a Lewis acid) suggests the possibility that it can enter the mitochondrial membrane if a negative-inside potential is applied. To apply a potential, we operate as follows: nonrespiring mitochondria were resuspended in a medium with a low K^+ concentration (60 μM final concentration) while the potassium concentration in the matrix is about 0.1 M. Since the mitochondria are not permeable to potassium, an outward K^+ flow is recorded by a selective potassium electrode only providing the presence of both excess valinomycin (the potassium carrier) and a positive counterion which ensures the charge balance by entering the mitochondria (21, 22). As Fig. 1a shows, $(Et)_3$-Pb^+ acts as a counterion since a significative K^+ efflux is observed only when $(Et)_3$-Pb^+ is added (and in the presence of valinomycin). For comparison, Fig. 1b shows that Ca^{2+} can be a counterion since also Ca^{2+} uptake is potential-driven and the transport through the membrane occurs by a selective Ca^{2+} carrier (21, 22). Figure 1c shows that addition of $(Et)_4$ Pb to the mitochondria containing valinomycin does not induce (a significant) K^+ efflux since $(Et)_4$ Pb does not form any lipophilic cation in solution. These evidences indicate that the measure of K^+ efflux is a measure of lipophilic cation uptake.

In Fig. 1a the K^+ efflux initial rate is the slope of the K^+ concentration against the time when the alkyl compound is added. As Fig. 2 reports, the initial rate is proportional to the concentration of added alkyl metal; in fact the influx of alkyl metal uptake is the rate-limiting step in the whole transport mechanism. This statement can be easily demonstrated by measuring the potassium efflux at any organometal concentration with a fixed amount of valinomycin (2 nmol/mg protein as indicated under Materials and methods). If the rate-limiting step is the rate of organometal influx, this rate will not be modified by enhancing the amount of added valinomycin; this is the situation that we observed in our experiments.

The procedure allows one to measure the rate of uptake of any compound for any concentration as shown in the case of $(Bu)_3$-Sn-Cl in the Fig. 2. The EC_{300} values reported in Table 1 compare the transport rates for all tested compounds.

When several organometal cations are present in solution, the electrode response is additive. For this reason, we compared the electrode responses with the toxicological responses of the *in vivo* red killifish (*Orizies latipes*) test, which gives an overall toxicological response. The logarithms of the 48-h LC_{50} values reported in the literature, according to the OECD test guideline (24), have been compared in linear regression analysis to the logarithms of the EC_{300} values arising from our test. Plotting Log EC_{300} vs Log LC_{50} gave a correlation coefficient (r) of 0.92, a slope of 0.234 and the intercept in the Y axis of -3.57. Inserting in this calculation the LC_{50} of $(Me)_3$-Hg-Cl which is reported only for the rainbow trout (25), the correlation is 0.90. These results suggest: (1) The uptake rate of organometals by the mitochondria can be utilized to define an *in vitro* toxicity test which reproduces the responses of *O. latipes* to the organometal compounds for a wide range of concentrations. (2) The good correlation (r = 0.92) suggests that, as proposed by Bragadin *et al.* (19), the prevailing toxic effect in the whole organism (*O. latipes*) could be due to a potential-driven uptake.

TABLE 1

Concentration Necessary to Obtain a Rate of 300 nmol K^+/mg/min (EC_{300} values) and the 48-h LC_{50} Values as Reported in the Literature for the *in Vivo* Test: *Orizies latipes*

Chemical compound	EC_{300} values (μM)	LC_{50} values (μM)
Bu_3-Sn-Cl	24 ± 2	0.111
Bu_2-Sn-Cl_2	158 ± 11	19.1
Bu-Sn-Cl_3	138 ± 10	135
Me_3-Sn-Cl	144 ± 11	28.2
Phe-Sn-Cl_3	158 ± 10	361
Phe_2-Sn-Cl_2	125 ± 10	89
Phe_3-Sn-Cl	36 ± 3	0.166
Me_2-Sn-Cl_2	160 ± 11	27.3
Ciclohexyl$_3$-Sn-Cl	50 ± 4	
Pro_3-Sn-Cl	85 ± 6	
Et_3-Sn-Cl	162 ± 11	
Bu_3-Pb-Cl	7 ± 0.5	
Me_3-Pb-Cl	140 ± 10	
Et-Hg-Cl	68 ± 5	
Me-Hg-Cl	11 ± 1	0.213

Note. The values of LC_{50} for Me-Hg-Cl are those reported for the 96-h rainbow trout test. The standard deviations values for the EC_{300} values are obtained on the basis of the standard deviations of the slope of the plots V_K^+ vs organometal concentration (see Fig. 2). Phe, phenyl; Me, methyl; Et, ethyl; Pro, propyl; Bu, butyl.

It can be emphasized that nonrespiring mitochondria are utilized. In principle, since not only mitochondria contain potassium in the matrix (which generates the driving force for organocation uptake), other membranous systems such as erythrocytes and liposomes can be utilized as biosensors.

Interferences in the measurement could be due to the presence of phenols in solution. Since phenols induce proton permeability (21), they can cause a potassium efflux. Such an interference, however, can be avoided by operating in the presence of 2% albumin, which complexes phenols (23).

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Analysis of Src Kinase and Protein Kinase C Activity by Capillary Electrophoresis and Laser-Induced Fluorescence[1]

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Conventionally, protein kinase assay has involved the phosphorylation of substrate peptides with [γ-^{32}P]ATP and the transfer of ^{32}P-labeled substrate peptide onto a paper disk (such as phosphocellulose paper) followed by scintillation counting. However, this method has its limitations such as reproducibility or safety. Recently, capillary electrophoresis (CE)[3] has become a powerful analytical system for the detection of a wide varieties of molecules from small organic molecules to macromolecules such as DNA and protein (1). Dawon *et al.* have established a CE-based protein kinase assay (2). However, its detection sensitivity is not satisfactory using UV detector. Therefore, it is tempting to establish a CE method for protein kinase assay with a more sensitive detector such as laser-induced fluorescence (LIF).

Recently, a diode laser emitting at a wavelength of 635 nm was incorporated in a CE/LIF detector system (Beckman). Also, an amino-reactive fluorescent cyanine dye, Cy5 (λ_{max} = 652 nm), was frequently used to label analytes including DNA for CE/LIF (635 nm) detection (3). Cy5 has a very reactive succinimidyl ester group which can be easily attacked by aliphatic primary amine (pH>pK_a ~9–10) (which is abundant in peptides and proteins) or hydroxyl group at neutral or alkaline pH (4). Taken together, it is feasible to develop a sensitive CE/LIF system for detection of Cy5-labeled protein kinase substrates and their phosphorylation products.

Two of the important protein kinase, PKC and Src kinase, were selected to establish CE/LIF analysis of protein kinase activity. PKC is a Ca^{2+}/phospholipid-dependent serine/threonine kinase, whereas Src is an protein tyrosine kinase. In this paper, synthetic substrate peptides of protein kinase C and Src kinase were labeled with Cy5 and used for the kinase reaction followed by CE/LIF analysis.

Instrumentation and electrophoretic conditions. A P/ACE 5000 CE analyzer (Beckman) equipped with a 2.5-mW diode laser emitting at 635 nm and a LIF detector were used. A diode array detector (wavelength range: 195–600 nm) was also used for detecting the unlabeled substrate peptide. Uncoated capillary columns (75 mm i.d.) of 47 cm length (40 cm to detector window) were assembled in the LIF cartridge format. Before the run, the column was rinsed with 0.5 N HCl followed by 0.1 N NaOH and distilled water. The column was filled with electrophoresis buffer by a high-pressure (15 psi) rinse of nitrogen for 1.5 min. The samples were then injected by low-pressure (0.5 psi) nitrogen for 2 s and separated at 12

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[3] Abbreviations used: CE/LIF, capillary electrophoresis/laser-induced fluorescence; DMSO, dimethyl sulfoxide; PKC, protein kinase C; RMT, relative migration time.

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