APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1986, p. 1099–1103
0099-2240/86/051099-05$02.00/0
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Vol. 51, No. 5

Bioaccumulation of Germanium by *Pseudomonas putida* in the Presence of Two Selected Substrates

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Received 1 July 1985/Accepted 31 October 1985

The uptake of germanium by *Pseudomonas putida* ATCC 33015 was studied in the presence of catechol or acetate or both as representative substrates differing in their ability to form complexes with this element. The bacteria were taken from a batch culture grown on acetate as the sole carbon source. Cells introduced into a medium containing germanium and either catechol or a mixture of catechol and acetate accumulated germanium in a biphasic way. After a lower level of accumulation that corresponded to the value obtained in the presence of acetate was reached, a further increase in the germanium content up to a higher saturation level was observed. The appearance of the second step of accumulation, which corresponded to the linear degradation of catechol, proved that catechol facilitated the transport of germanium into the cells through the nonspecific uptake of the germanium-catechol complex by an inducible catechol transport system.

Germanium, an element belonging to group 4A of the periodic system (along with carbon, silicon, tin, and lead), is widely found in waste products of coal and coke technology. Coal ash may contain 20 to 280 mg of Ge kg^{-1} (16). Although there is no definite evidence for the mutagenicity of germanium compounds, it was recently reported (10) that germanium dioxide (GeO_2) behaved as a potent antimutagen on frameshift-type reverse mutations induced by 3-amino-1-methyl-5H-pyrido[4,3-b]indole in *Salmonella typhimurium* TA98 and TA1538. Some germanium compounds are also known for their application as therapeutic agents in medicine, e.g., an organogermanium compound served as a significant interferon-inducing agent (9). Some microalgae, such as *Chlorella* and *Spirulina* spp., which were able to accumulate up to 4 mg · g of $cells^{-1}$, might be a potent source of health food with a therapeutic efficacy for humans (18).

Germanium can be recovered from wastewaters of coke technology by the use of microorganisms. It was previously reported that a mixed population of activated sludge microorganisms accumulated significant amounts of germanium during the biodegradation of phenolic substrates present in coke wastewaters (J. Chmielowski and C. Olczak, Abstr. 10th FEBS Meeting, Paris, France, abstr. no. 1119, 1975). Germanium in the form of free ions (it should be noted that germanium exists in this form when the medium is devoid of the growth substrate or when the substrate does not complex with germanium) was accumulated only to a much smaller extent. Accumulation was enhanced in the presence of, e.g., catechol, which bound one Ge atom per two (2) or three (1, 13) molecules of ligand. The results obtained with a mixed population of microorganisms justified the continuation of experiments on germanium bioaccumulation by pure cultures of bacteria. A wild-type strain of *Pseudomonas putida* was chosen as the one which was dominant in the mixed population of the phenolic activated sludge. Pure cultures of *Pseudomonas* sp. were found to be able to accumulate germanium and other nonessential metals when grown in an environment supplemented with organic substrates which were able to complex with this element (11; J. Chmielowski, A. Danch, and B. Kłapcińska, Abstr. 12th FEBS Meeting, Dresden, German Democratic Republic, abstr. no. 3940, 1978; J. Chmielowski, Abstr. 14th FEBS Meeting, Edinburgh, Scotland, 1981). Germanium is a suitable metal for the study of transport phenomena in *P. putida* as it does not affect the metabolic activity of the cells, i.e., neither respiration nor catechol biodegradation is altered (data not shown).

The transport of metal ions into bacterial cells can occur via one of the following mechanisms: (i) transport via carriers specific for nutritionally essential ions, e.g., Mn^{2+}, Mg^{2+}, and Ca^{2+}; (ii) specific transport of metals complexed with specifically exuded low-molecular-weight ligands (termed siderophores) through the cognate membrane receptors; (iii) nonspecific transport of metal ions complexed with substrates serving as carrier molecules through a transport system specific for these substrates; (iv) passive immobilization of metal ions within the cell envelope, owing to their binding by charged groups. Metal ions can also be less firmly immobilized within bacterial slimes by surface sorption (3, 8, 14).

The purpose of this study was to provide evidence that germanium is transported into bacterial cells as a complex with an aromatic substrate. It was presumed that this could be accomplished by comparing the course of germanium uptake in the presence of catechol or acetate. The latter compound does not complex with germanium. The biodegradation of catechol by *Pseudomonas* sp. is known to be mediated by an inducible enzyme system (7, 12).

MATERIALS AND METHODS

Organisms and culture media. *P. putida* ATCC 33015 was obtained from P. A. Williams (University College of North Wales, Bangor, United Kingdom). This strain carried a TOL plasmid (17). The bacteria were grown in a standard mineral medium (15) supplemented with 10 mM sodium acetate as the sole source of carbon and were maintained at 30°C on a reciprocating shaker. The mineral medium contained the following (in grams $liter^{-1}$): KH_2PO_4, 1.0; $(NH_4)_3PO_4 \cdot 3H_2O$, 0.325; NaCl, 0.05; $CaCl_2 \cdot 6H_2O$, 0.098;

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$FeCl_3$, 0.01; and $MgSO_4 \cdot 7H_2O$, 0.05. The pH was adjusted to 7.3. The organisms were maintained by being subcultured daily in fresh medium.

Bacteria harvested from this growth medium by centrifugation for 15 min at 5,000 rpm (5,200 × *g*) were used as a source of inocula for experiments on germanium bioaccumulation. In these experiments the mineral medium was supplemented with 2 mM GeO_2 (Johnson, Matthey and Co., Ltd.) and catechol (4 mM), sodium acetate (5 or 10 mM), or a mixture of both of these substrates.

Analytical methods. The catechol content in the medium was estimated spectrophotometrically (Beckman DK-2A spectrometer) by measuring the A_{275} in a 1-cm cell by using a standard curve prepared for a series of solutions of known catechol content. The dry weight of the organisms was determined by collecting cells from a 50-ml sample on a predried and weighed Coli-5 filter (0.45-μm pore size) and drying them to a constant weight at 105°C (6). The growth of bacteria was monitored turbidimetrically at 550 nm by using a Specol spectrocolorimeter equipped with a nephelometric attachment. The dry weight of the culture biomass was calculated from the standard curve by relating the turbidity of the culture biomass to the dry weight of the cells.

Determination of the uptake of germanium. All the germanium solutions used in the bioaccumulation experiments were labeled with ^{71}Ge (OPIDI, Świerk, Poland), which had a half-life of 11.4 days and an initial activity of 3.7 MBq. A solution containing $^{71}GeO_2$ was added to medium supplemented with GeO_2 in an amount that depended on the actual activity of the radioisotope. The germanium content in the cells and in the medium was determined radiometrically (4). At various times 10-ml samples were withdrawn from the growth medium, and the cells were harvested by centrifugation at 5,000 rpm (5,200 × *g*) for 15 min at 4°C. The pellet was washed with an equal volume of mineral medium, suspended in 10 ml of distilled water, and sonicated twice for 30 s at 0°C in an ultrasonic disintegrator (Labsonic 1510) with a frequency of 20 kHz. Two separate 4-ml samples of the sonicated suspension were transferred to glass vials, and 6 ml of the gelling scintillator Insta-gel (Packard Instrument Co., Inc., Rockville, Md.) or scintillation fluid (2,5-diphenyloxazole [PPO], 5.5 g; 1,4-bis(5-phenyloxazolyl) benzene [POPOP], 0.1 g; Triton X-100, 334 ml; toluene, 666 ml) was added to each vial. The samples were counted in a Beckman LS 3133P liquid scintillation counter with ^{71}Ge as an internal standard. The level of cell-associated germanium was expressed as micromoles of Ge gram (dry weight) of $cells^{-1}$.

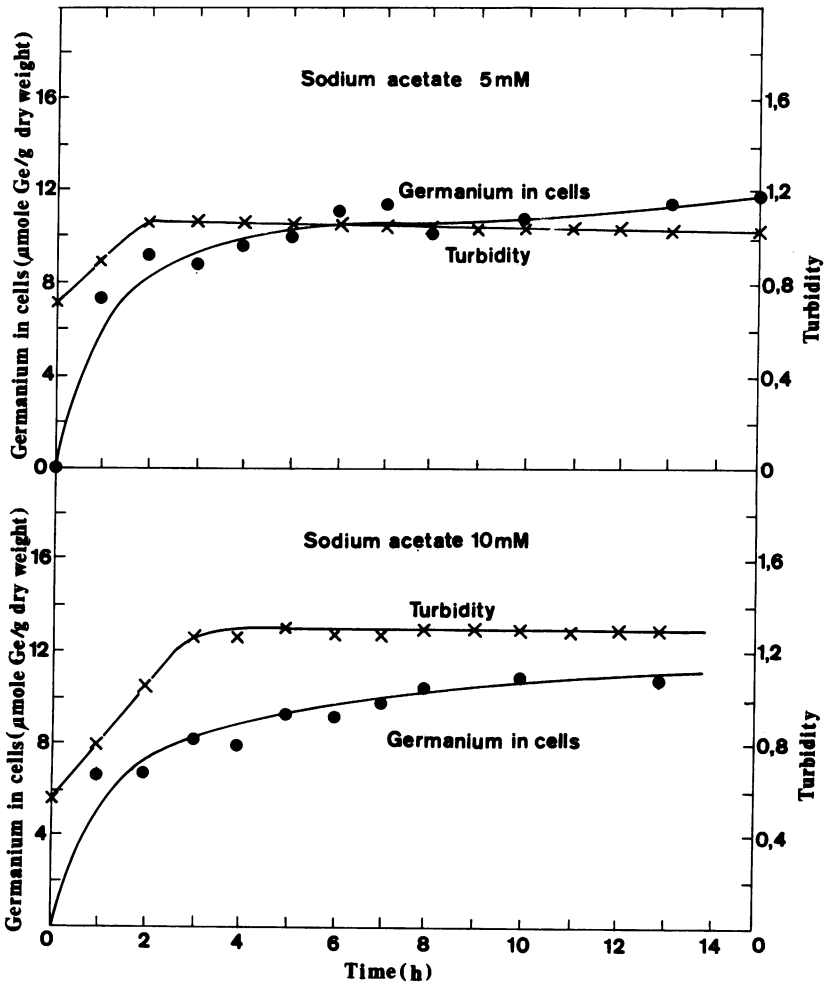

FIG. 1. Germanium accumulation and growth of *P. putida* ATCC 33015 in the presence of 5 or 10 mM acetate. The initial germanium concentration was 2 mM.

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RESULTS AND DISCUSSION

The bioaccumulation of germanium by *P. putida* cells was investigated by observing the changes in the germanium content in bacterial cells during aeration in a mineral medium containing GeO_2 (2 mM) and sodium acetate (5 or 10 mM), catechol (4 mM), or a mixture of both of these substrates. The bacteria for bioaccumulation experiments were taken from a batch culture grown on acetate as the sole carbon source. It is known that acetate is readily utilized by most *Pseudomonas* species (5). In *P. putida* the acetyl group is one of the end fragments of the catechol cleavage pathway (7, 12). Therefore, it was assumed that acetate would act as an end-product repressor and would reduce the rate of synthesis of the enzymes that mediate catechol dissimilation.

When *P. putida* was grown in the standard mineral medium containing 2 mM GeO_2 and 5 mM acetate, the uptake of germanium by the cells rapidly achieved a plateau value (about 10 μmol of Ge · g [dry weight] of cells^{-1}) (Fig. 1). Similar bioaccumulation results were obtained when the medium was supplemented with 10 mM acetate (Fig. 1); the only difference was a higher growth yield in the latter case. This result indicated that the level of germanium bioaccumulation was not affected by substrate (acetate) uptake.

The bioaccumulation of germanium from medium containing 2 mM GeO_2 and 4 mM catechol exhibited a biphasic course (Fig. 2). During the first step of the uptake, the germanium content in the cells rapidly reached the level obtained in previous experiments with acetate; it then increased to a higher saturation value. This second step of the uptake began when approximately half of the catechol was metabolized during an intensive linear (zero-order) dissimilation of this substrate. The ultimate value of germanium uptake was found to be as high as about 22 μmol of Ge · g (dry weight) of cells^{-1}.

A similar biphasic course of germanium bioaccumulation was observed when the bacteria were grown in medium containing both catechol (4 mM) and acetate (5 or 10 mM). As in the previous experiment, after the germanium content reached the level corresponding to the value achieved in the presence of acetate (about 8 μmol of Ge · g [dry weight] of cells^{-1}), a further increase in the germanium content in the bacterial biomass up to a higher saturation value (about 20 μmol of Ge · g [dry weight] of cells^{-1}) was observed when 5 mM acetate was used (Fig. 3). The ultimate value of germanium uptake did not, however, exceed the value reached in the experiment with catechol as the sole growth substrate; the only difference was that with both substrates the biodegradation of catechol and the bioaccumulation of germanium started after a longer lag. This effect was even more pronounced in the case of a higher initial acetate concentration (10 mM) (Fig. 4), owing to the longer dissimilation time of acetate when used at a higher concentration. In the two-substrate system, the second step of germanium bioaccumulation began after the utilization of approximately half of the catechol, coinciding with the early stationary phase of the growth cycle.

From the shape of the growth curves it was concluded that the apparently biphasic germanium uptake might be related to the consecutive dissimilation of the growth substrates present in the medium. No distinct diauxie was observed in the growth curves. However, two marked linear segments differing in slope and corresponding to the logarithmic

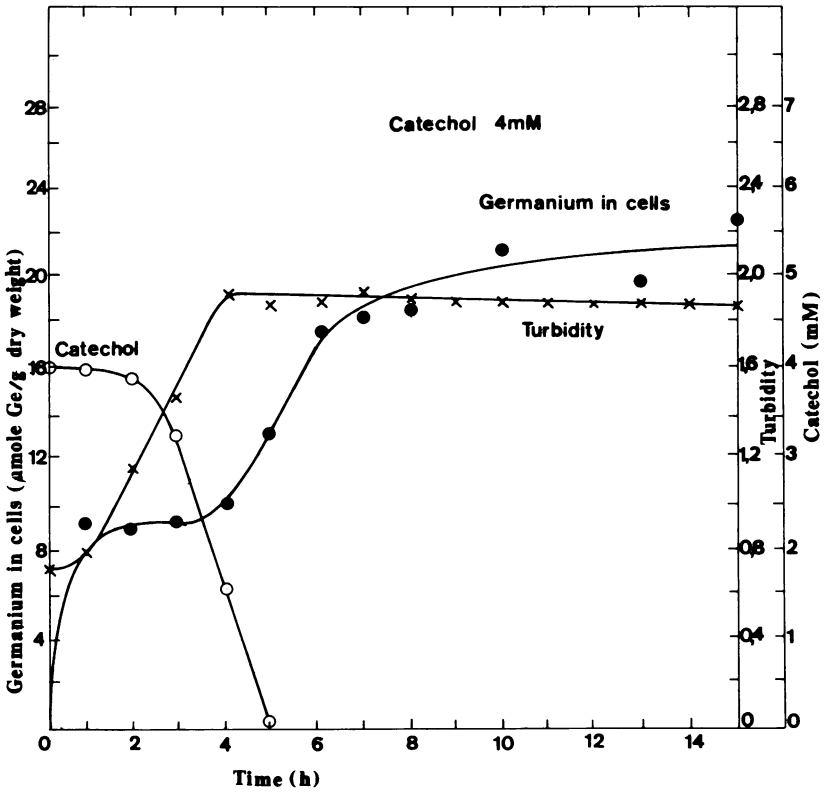

FIG. 2. Germanium accumulation, catechol degradation, and growth of *P. putida* ATCC 33015 in the presence of 4 mM catechol. The initial germanium concentration was 2 mM.

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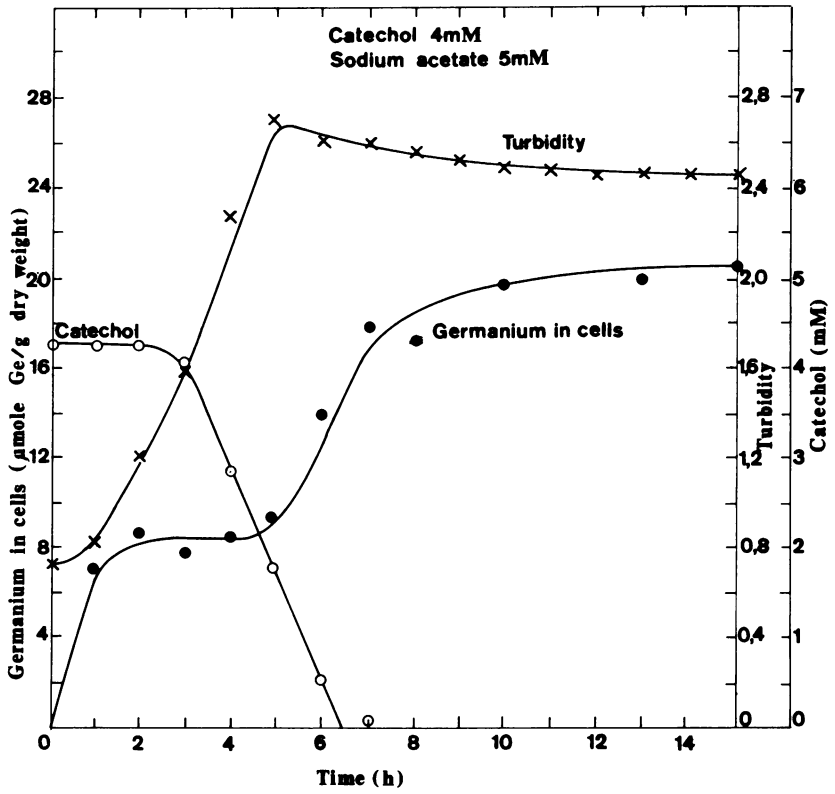

FIG. 3. Germanium accumulation, catechol degradation, and growth of *P. putida* ATCC 33015 in the presence of 4 mM catechol and 5 mM acetate. The initial germanium concentration was 2 mM.

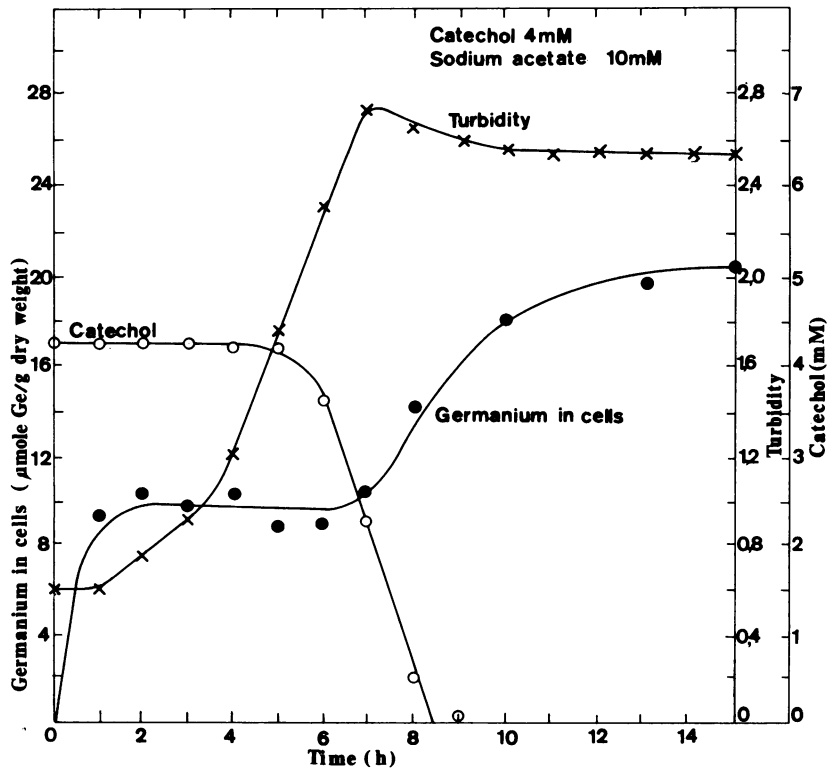

FIG. 4. Germanium accumulation, catechol degradation, and growth of *P. putida* ATCC 33015 in the presence of 4 mM catechol and 10 mM acetate. The initial germanium concentration was 2 mM.

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growth of bacteria on acetate and then on catechol could be distinguished in the growth curves (Fig. 4). Growth at the expense of catechol required fully induced levels of all the enzymes of catechol catabolism; therefore, it was concluded that these enzymes should already be synthesized during the dissimilation of acetate. No diauxie of growth was observed; i.e., there was no lag phase which would correspond to the induction of the catechol cleavage enzymes after the depletion of acetate from the medium. This result also indicates that acetate was not a very effective repressor of the synthesis of these enzymes. When acetate was the sole growth substrate, however, inhibition of the synthesis of the inducible enzymes involved in catechol catabolism was sufficient to allow us to elucidate the probable mechanism of germanium transport into bacterial cells based on the data obtained from germanium uptake experiments.

The biphasic course of germanium bioaccumulation supported our previous presumption that catechol facilitated the transport of this element into the cell (Chmielowski, Abstr. 14th FEBS Meeting), provided that the bacteria were induced to degrade this aromatic substrate. Under the experimental conditions (pH 7.3), catechol spontaneously bound germanium into a complex (2, 13); however, as a result of bioaccumulation less germanium was bound by the organisms than was expected, assuming that the whole complex entered the cell. The germanium content in the medium decreased by only about 5% even after the complete removal of catechol by the bacteria. It seems probable that, owing to the high activity of inducible enzymes of the catechol cleavage pathway, the degradation of free catechol present in the medium took place. This could result in a change in the stability of the complex (it will be stabilized by the excess germanium as catechol disappears from the medium) and might enable the uptake of the complex in the undissociated form into the cell via the inducible catechol transport system, which does not discriminate between free and complexed catechol. Thus, the uptake of germanium from a medium containing germanium complexed with readily dissimilated substrates may be considered an example of a nonspecific intracellular accumulation of a metal (or a metalloid) by microorganisms (Chmielowski, Abstr. 14th FEBS Meeting). The intracellular accumulation of germanium in *P. putida* cells was revealed in a separate work (11). It was accompanied by surface sorption and surface binding of this element to the cell envelope; the extent of these corresponded to the uptake values obtained in the presence of acetate or, during the first step of accumulation, in a medium supplemented with catechol or mixed substrates.

ACKNOWLEDGMENT

We thank P. A. Williams (Department of Biochemistry and Soil Science, University College of North Wales, Bangor, United Kingdom) for his gift of the bacterial strain.

This work was partially supported by contract MR-II-17.

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