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Phosphoesterase Activity and Phosphate Release from Tributyl Phosphate by a *Citrobacter* sp.

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ABSTRACT

Tributyl phosphate (TBP) and other alkyl phosphates represent a class of persistent organophosphorus compounds of widespread use. Biodegradation of the phosphotriesters is postulated to occur through sequential hydrolytic cleavages via the phosphodiester and monoester intermediates to alcohol and inorganic phosphate (P_i). Immobilized cells of a *Citrobacter* sp. liberated P_i upon challenge with TBP but the reaction was short-lived. In vitro studies with purified phosphomonoesterase (phosphatase) used ^{31}P nuclear magnetic resonance to demonstrate P_i transfer onto ethanol (phosphotransferase activity). This suggested that in vivo the onset of a futile phosphohydrolytic and transphosphorylation cycle would limit the extent of phosphate production. A mutant deficient in the transphosphorylating phosphomonoesterase showed an extended release of P_i under challenge with TBP that was not subject to the complete and premature reaction termination that precluded application of the parent strain to possible industrial processes for alkyl phosphate biodegradation.

Index Entries: Tributyl phosphate degradation; *Citrobacter* sp.; phosphatase; transphosphorylase; nuclear magnetic resonance.

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INTRODUCTION

Tributyl phosphate (TBP), a solvent and plasticizer (*1*), finds special use in nuclear fuel reprocessing where, despite in-process recycle, waste TBP loaded with residual uranyl ion presents a final disposal problem. A biotechnological approach to this problem demonstrated apparent biodegradation of TBP by a *Citrobacter* sp. (*2*); the liberated inorganic phosphate was used to desolubilize heavy metals (M^{2+}) at the cell surface as $MHPO_4$ (*3*). The potential for a coupled TBP degradation and uranium bioaccumulation treatment process is apparent; the present study addresses the possible enzymatic processes governing the sequential hydrolysis reactions leading ultimately to the production of inorganic phosphate (P_i) and *n*-butanol from TBP via the postulated intermediates dibutyl phosphate (DBP: phosphate diester) and monobutyl phosphate (MBP; phosphate monoester). Evidence for such hydrolytic cleavage of alkyl phosphates has been obtained previously (*4*), but few studies have been performed on TBP biodegradation *per se* (*see 5*).

An earlier report demonstrated liberation of phosphate by a TBP-challenged *Citrobacter* sp. (*2*) that had been previously shown to accumulate heavy metals extensively as $MHPO_4$ (*3*). The role of the phosphomonoesterase in TBP breakdown was largely discounted (*2*). A labile phosphodiesterase was also identified but a role for this enzyme in the breakdown of TBP was discounted on the basis of a requirement of the enzyme for Mn^{2+} (inhibitory to TBP hydrolysis) and competition studies (*2*).

The present investigation addresses the production and activities of the phosphoesterases postulated to be responsible for TBP breakdown in vivo. Evidence for the correlation of phosphotriester and monoester breakdown was sought. In vitro studies using ^{31}P nuclear magnetic resonance (NMR) and subsequent investigations using a monoesterase— deficient mutant suggested that phosphotransferase activity may interfere with phosphate liberation from TBP. The implications of this possibility are discussed.

METHODS

Microbial Strains

The parent strain, *Citrobacter* sp. N14 and phosphomonoesterase-deficient mutant M1 were as described previously (*2*). The mutant, obtained by ethyl methane sulfonate mutagenesis, had a phosphomonoesterase activity of less than 5% of that of the parent strain using either whole cells or cell extracts (*2*). The phosphomonoesterase overproducing strain dc5c was obtained by a selection technique using Cu^{2+} to select for phosphatase overproducers; the phosphomonoesterase activity was increased by two- to threefold as compared to the parent strain N14 (*6*).

Media and Growth Conditions

The cells were routinely grown in tris-buffered glycerol-glycerol 2-phosphate minimal salts medium as described previously (2). Growth was performed at 30°C with aeration provided by agitation (<250 mL cultures) or by vigorous streams of sterile air (31 batches).

Preparation of Immobilized Cells and Phosphate Release Experiments

The 31 batches were harvested and immobilized in a polyacrylamide gel as described previously (2). The gel was shredded for use; each preparation provided material for ~50 × 1 g aliquots (1 g wet wt apiece; each aliquot contained approx 10–12 mg dry wt of cells). For each phosphate release experiment one 1 g aliquot was preequilibrated at 30°C in 27 mL of citrate buffer, pH 6.9. The reaction was initiated by the addition of substrate (glycerol 2-phosphate or TBP; 50 m*M*) to final concentrations of 2 and 5 m*M* of buffer and substrate, respectively; the final reaction volume was 30 mL. The flasks were shaken at 30°C. Samples (0.3 mL) of the perfusing mixture were withdrawn into 2.5*M* H_2SO_4; this did not elicit spontaneous hydrolysis of the substrates. Phosphoesterase activity was expressed as μmol of P_i product liberated g^{-1} of gelled material, with inorganic phosphate determined by a modified version of the Pierpoint method (7) vs inorganic phosphate standards similarly assayed. Phosphoesterase specific activity (unit) is defined as μmol P_i liberated g^{-1} of gelled material h^{-1}, or, for nonimmobilized cells as μmol P_i liberated mg bacterial $protein^{-1}$ min^{-1}.

Determination of Phosphoesterase Activity Using Free Cells

In some experiments cells were not immobilized but were harvested during growth to monitor phosphoesterase activity; details are given where appropriate. Here the harvested samples were separated from the medium by centrifugation, washed in isotonic saline (8.5 gL^{-1} of NaCl) and stored as pellets at 4°C until use (generally overnight). For assay the cells were resuspended to an OD_{600} of 0.3–0.4 (Perkin-Elmer spectrophotometer) in citrate buffer and assayed as described for immobilized cells, except that the reaction was stopped by rapid chilling and removal of the cells by centrifugation prior to assay of the liberated P_i by the Pierpoint method as above.

Special Precautions for Phosphate Release Experiments

All experiments were done in "dedicated" glassware pretreated to remove phosphate either by washing in H_2SO_4 (2.5*M*) or soaking in

"Decon 90" (2% v/v aqueous solution: Decon Laboratories, Hove, England) followed by copious rinsing in glass distilled water. Solutions for assay were prepared using "milli Q" treated water in dedicated glassware. The background P_i contamination was minimal. All assay determinations were in triplicate, determined against phosphate-unsupplemented "blanks" and P_i standards (6 replicates apiece).

Purification of Phosphomonoesterase

The *Citrobacter* phosphomonoesterase (phosphatase) was purified to homogeneity as described previously (*2*). The phosphomonoesterase activity was attributable to two very similar isoenzymes designated CPI and CPII (*8*). Purification of phosphodiesterase and putative phosphotriesterase was not undertaken in this preliminary study since the specific activities of the enzymes in the cells were low and data on the stability of the enzymes in vitro were not available. During purification phosphatase activity was measured by the liberation of *p*-nitrophenol from *p*-nitrophenyl phosphate (pnpp), with protein assayed in whole cells by the Lowry method and in enzyme preparations by the method of Bradford with bovine serum albumin as standard (*2,8*).

NMR Spectroscopy

Phosphomonoester cleavage and product generation were followed in vitro using ^{31}P nuclear magnetic resonance spectroscopy (^{31}P NMR), in a multinuclear NMR spectrometer designed and built in the Department of Biochemistry, University of Oxford. The system was based on Oxford Instruments 8.46T (360 MHz ^1H) superconducting wide-bore magnet. ^{31}P NMR spectra were obtained at 145.8 MHz. All spectra were taken with 2.5 mL samples in a 10 mm diameter tube fitted with a capillary insert containing MDPA (methylene diphosphonic acid: 10 m*M*) dissolved in D_2O as an internal field frequency lock. The chemical shift of 85% phosphoric acid was used as a reference for measuring ^{31}P chemical shifts. Each ^{31}P spectrum was obtained with a simple pulse and collect sequence using a 36° excitation pulse (12 μs) and an interpulse delay of 1.2 s. The spectra were obtained at room temperature without spinning. For each spectrum 400 scans were collected.

The reaction was set up and done in the NMR tube (2.5 mL final volume). Purified phosphatase (4.4 and 2.2 μg for isoenzymes CPI and CPII, respectively) was preequilibrated in 200 m*M* MOPS buffer, pH 7.0, and the reaction was initiated by the addition of *p*-nitrophenyl phosphate (pnpp, substrate: to 2.7 m*M*). After mixing the tube was transferred to the NMR spectrometer and the reaction was monitored by ^{31}P chemical shift. In some experiments ethanol was incorporated: (2.7*M* final concentration). Butanol was inappropriate for NMR experiments; ethanol provided a water soluble alternative. Alkyl phosphate appearance was monitored vs

Table 1
Phosphate Release from Glycerol 2-Phosphate and TBP by PAG-Immobilized Cells[a,b]

	Parent strain N14					Phosphomonoesterase overproducer dc5c	
	Batch I	Batch II	Batch III	Batch IV	Batch V	Batch I	Batch II
P_i release from glycerol 2-phosphate (A)[a]	9.1	22	27	31	22	45	78
P_i release from TBP (B)[a]	0.36	0.75	0.75	1.37	0.83	0.58	1.36
Calculated rate of TBP cleavage (C)[c]	1.08	2.25	2.25	4.11	2.49	1.74	4.08
Ratio of A/C	8.4	9.8	12.0	7.5	8.8	25.9	19.1

[a] μmol phosphate released $h^{-1}g^{-1}$ of gelled material (30 mL batch incubations).
[b] For tributyl phosphate the concentration of TBP was probably less than 5 m*M* owing to the low water solubility of this compound. The challenge solution was shaken vigorously throughout the experiment.
[c] For the triester substrate cleavage of three phosphoester bonds is necessary to liberate inorganic phosphate (P_i). The rate of reaction against the triester is thus taken as threefold greater than that detectable by phosphate release; the data shown are corrected accordingly and the rate of phosphate release from glycerol 2-phosphate vs that observed on TBP addition was 9.3 ± 0.77 (5) for strain N14, and 22.5 (2) for strain dc5c.

a commercially obtained standard of a mixture of mono and diethyl phosphate (38%: 60% v/v; Tokyo Kasei Kogyo Co. Ltd., via Fluorochem Ltd). The composition of the mixture was confirmed by the manufacturers, on request.

RESULTS

Phosphate Release from Glycerol 2-Phosphate and Tributyl Phosphate by Polyacrylamide Gel (PAG)-Immobilized Cells

Initial studies employed *Citrobacter* strain N14 and a mutant derivative dc5c that overproduced phosphomonoesterase ~2.5–3-fold as compared to the parent strain (*6*). The gelled cells were challenged with substrate (glycerol 2-phosphate or TBP; 5 m*M*); the respective rates of P_i release promoted by the two substrates are shown in Table 1. The correlation coefficient of five independent batches of strain N14 was 0.886, which is significant at $p = 0.95$ (Table 1). A similar overall correlation was seen for strain dc5c, but since the data were from two batches only no further calculations were attempted. Previous studies have suggested the

participation of more than one enzyme (2); this conclusion is supported by the data of Table 1. No difference was seen in the rate of phosphate release on TBP addition between the two strains although monoesterase activity was clearly overexpressed in the mutant strain (Table 1); presumably the mutation was monoesterase specific.

The data of Table 1 show the comparative initial rates of phosphate release from glycerol 2-phosphate and TBP by the PAG-immobilized cells. Phosphate release was not sustained but ceased after about 90 min, with a maximum total of phosphate released of about 2 μmol of P_i/g of gelled material (2) corresponding to a solution concentration of P_i of about 0.067 m*M*. Since the concentration of TBP in water is maximally about 1 m*M* (*1*) (incorporation of higher concentrations would increase the amount in the nonaqueous dispersion) it could be suggested that the concentration of aqueous TBP becomes limiting. However since the concentration of TBP cleaved would be less than 7% of that nominally available (1 m*M*) this explanation is unlikely; furthermore; sonication to further disperse the TBP droplets in order to increase the TBP/water interface area did not enhance TBP breakdown (S. Owen and L. E. Macaskie, unpublished). For an industrial process a sustained activity would be necessary; this consideration prompted an investigation into some possible reasons for premature termination of the reaction.

The Effect of Cell Aging on Phosphate Release from TBP

Previous studies on the *Citrobacter* phosphohydrolases demonstrated a phosphodiesterase with activity sustained for a few hours only (2) and the possibility of a similar lability of the putative triesterase was considered. However, the activity was fairly stable in stored, immobilized cells with a loss of phosphate release of ~30% after 1 month (retention of activity over 1 mo in storage was 71.4 and 69.8% for two batches). The activity is thus comparably stable to the monoesterase (activity half-life approx 2 mo: 9) and enzyme lability would not account for the loss of activity which occurred over 2 h in the batch incubations. Furthermore, previous studies using immobilized cells indicated that TBP-promoted phosphate liberation could be restored on washing the gel (*10*).

Transphosphorylase Activity of Purified *Citrobacter* Phosphomonoesterase

The above experiments suggest that end-product inhibition (butanol) might have been responsible for the loss in activity seen. It was assumed that at a probable ratio of 3:1 to excess over the liberated phosphate the concentration of butanol in the vicinity of the hydrolytic enzyme(s) might be substantial. No attempt was made to quantify this in vivo. An in vitro test system was developed to demonstrate that transphosphorylation of liberated phosphate onto *n*-butanol via phosphomonoesterase activity

might account for the cessation of appearance of phosphate product. In these experiments ethanol was used as a water-soluble analog of *n*-butanol, and possible recipient molecule for phosphate. These tests employed ^{31}PNMR to follow the hydrolytic and transphosphorylation reactions of purified *Citrobacter* phosphatase (isoenzymes CPI and CPII: 2) using *p*-nitrophenyl phosphate as a test substrate. The chemical shifts attributable to *p*-nitrophenyl phosphate (substrate), inorganic phosphate (primary product), and ethyl phosphate (proposed transphosphorylation product) are shown in Fig. 1A, B. The ethyl phosphate standard was a commercially available mixture of monoethyl and diethyl phosphate: no attempt was made to further purify the standard or to differentiate between the peaks; two peaks were clearly visible (Fig. 1B). Preliminary experiments established that in the absence of added ethanol the hydrolytic reaction visualised only inorganic phosphate as a product. Incorporation of ethanol into the reaction mixture gave a similar decrease in the concentration of substrate but a parallel production of peaks corresponding to inorganic phosphate and ethyl phosphate. Identical results were obtained for isoenzymes CPI (Fig. 1C,D) and CPII (Fig. 1E,F,G,H); with the latter a complete time course is shown. The reaction rate was dependent on the enzyme concentration. Thus the rate catalyzed by CPI was more rapid than that for CPII on the basis of substrate disappearance (Fig. 2A), corresponding to enzyme loadings of 4.4 and 2.2 μg, respectively. The product was approximately equally divisible into inorganic phosphate (Fig. 2B) and ethyl phosphate (Fig. 2C) for both isoenzymes, with slightly more rapid production of ethyl phosphate. The product stoichiometry was maintained throughout the reaction up to 16 min (Fig. 2B,C). More detailed kinetic studies were outside the scope of this investigation, and care should be exercised in the extrapolation of in vitro results to the gross effects seen in PAG-immobilized whole cells, but the data suggest the possibility of liberated butanol acting as a sink for liberated phosphate during TBP biodegradation; here the transphosphorylation reaction would compete with the P_i liberation, with a reduction in extracellular P_i appearance.

Further Studies Employing the Phosphomonoesterase-Deficient Mutant M1

The above hypothesis would suggest that in the absence of substantial phosphotransferase (i.e., phosphomonoesterase) activity, the rate of P_i liberation from TBP might be increased if it is assumed that the rate of removal of phosphate from the vicinity of the reaction is limited in the wild-type strain by the diffusional gradient out of the gel. Confirmatory studies utilised the monoesterase deficient mutant M1 (phosphatase activity ~4% of that the parent strain: 2). Some interbatch variation was seen; typical data from three batches are shown in Fig. 3. In the absence of substantial monoesterase activity (strain M1) both the rates and extents of phosphate liberation were increased in the mutant strain, with good

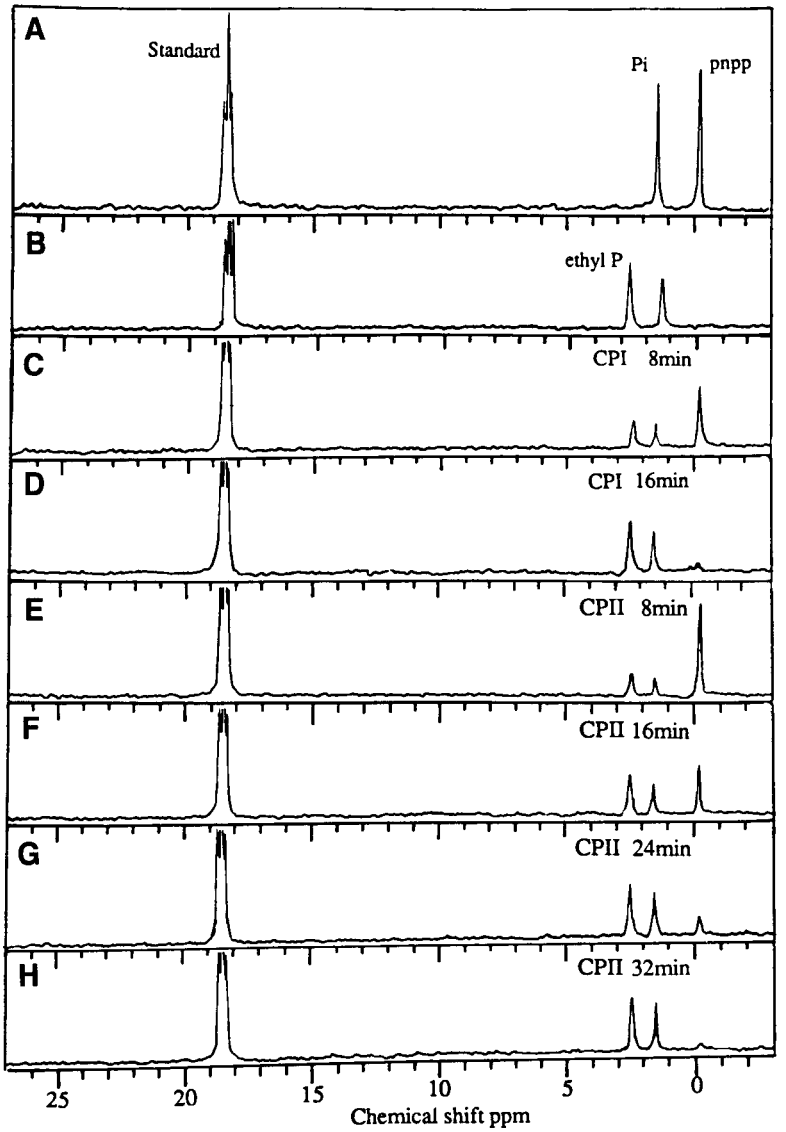

Fig. 1. ^{31}P nuclear magnetic resonance study of phosphohydrolase and phosphotransferase reactions catalyzed by purified *Citrobacter* phosphomonoesterase (isoenzymes CPI and CPII). The isoenzymes were suspended in buffer as described in Methods in the absence or presence of ethanol at a 1000-fold excess to substrate (*p*-nitrophenyl phosphate, pnpp). The reaction was initiated by the addition of substrate and followed in a NMR spectrometer as described in Methods, vs a standard of 85% phosphoric acid as a reference for measuring ^{31}P chemical shifts. (**A,B**) Standard peaks for *p*-nitrophenyl phosphate and inorganic phosphate, and monoethyl and diethyl phosphate (commercially obtained mixture: 38%:60%, *see text*. (**C,D**) Time course of the reaction using isoenzyme CPI (4.4 μg of protein per 2.5 mL incubation) shown at 8 min (**C**) and 16 min (**D**). (**E, F, G, H**) Time course of the reaction using CPII (2.2 μg of protein per 2.5 mL incubation) shown at 8 min (**E**), 16 (**F**), 24 (**G**), and 32 (**H**) min.

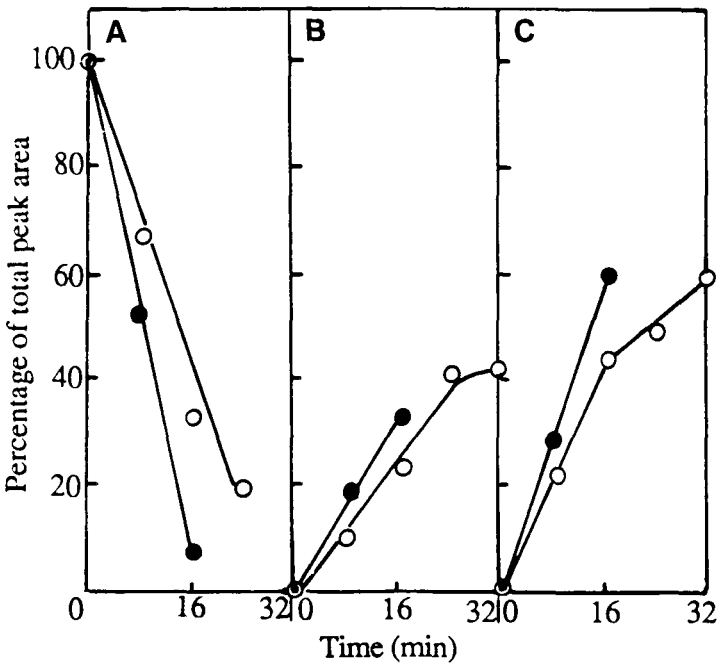

Fig. 2. Time course of *p*-nitrophenyl phosphate disappearance (**A**) and the appearance of the inorganic phosphate (**B**) and ethyl phosphate (**C**) products in the presence of ethanol. Integrated areas under the peaks are expressed as percentage of the total pooled peak area in each case. Filled symbols: CPI. Open symbols: CPII.

reproducibility. Pooled data from several replicate batches are shown in Table 2. Both the rate and extent of phosphate liberation were increased by 32% in the mutant strain; removal of competing transphosphorylation reactions is implicated in the enhancement of phosphate release from TBP-challenged cells.

DISCUSSION

Phosphotransferase activity by *Citrobacter* phosphatase (phosphomonoesterase) is suggested as one cause of interference in the production of inorganic phosphate by TBP challenged cells, and for bioprocess use the phosphatase deficient strain M1 would offer a significant advantage. Although the in vitro ^{31}P NMR data cannot be applied directly to the whole cells, the coappearance of inorganic phosphate and alkyl phosphate products (Fig. 2) suggest that even in the presence of substantial transphosphorylase activity inorganic phosphate should be produced alongside the transphosphorylation product. The rate of phosphate liberation should be approximately halved according to the data of Fig. 2.

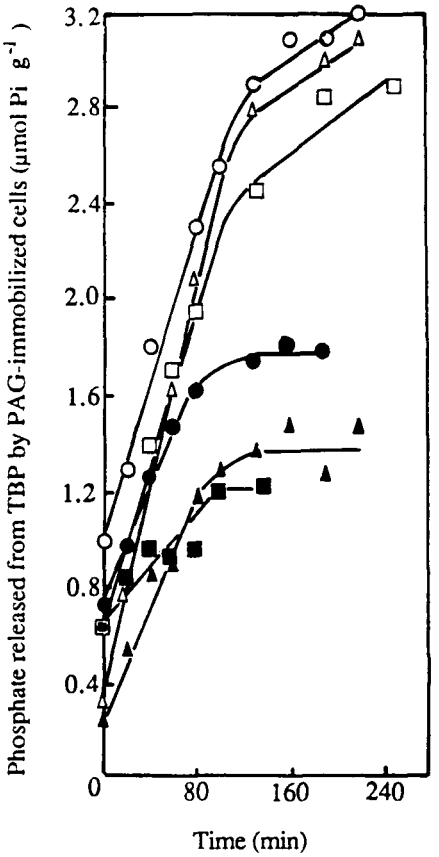

Fig. 3. Phosphate release by TBP-challenged cells of the parent strain N14 (filled symbols) and by the phosphomonoesterase (phosphatase)-deficient mutant M1 (open symbols). Data are shown for three independent batches (●, ▲, ■).

This was not seen in strain N14, where activity ceased after 90 min, although strain M1 did display the expected behavior (Fig. 3). These data suggest that while transphosphorylase activity may contribute a "nuisance" effect on phosphate liberation this does not constitute the only reason for the cessation of activity of the immobilized cells. A search for alternative mechanisms of inhibition has suggested that reaction intermediates such as dibutyl phosphate (DBP) may also exert inhibitory effects (R. E. Dick and L. E. Macaskie, unpublished) and future studies will attempt to quantify the relative contributions made by DBP product inhibition and transphosphorylation reactions. If reduced transphosphorylation activity is confirmed in mutant M1 this strain would serve as a useful tool for this aportionment.

Table 2
Phosphate Release from TBP
by *Citrobacter* sp. N14 and the Phosphomonoesterase-Deficient Mutant M1[a]

	Rates of phosphate release from TBP, μmol P_i h^{-1} g^{-1} of gelled material	
	Strain N14, parent	Strain M1, phosphomonoesterase-deficient
Batch I	0.425 ± 0.12 (*8*)	1.010 ± 0.049 (3)
Batch II	0.843 ± 0.095 (*10*)	1.090 ± 0.110 (4)
Batch III	0.700 ± 0.030 (7)	
Batch IV	1.130 ± 0.170 (3)	
Batch V	0.875 (2)	
	Time to termination of linear phosphate release, "cut off", min	
	Strain N14, parent	Strain M1, phosphomonoesterase-deficient
Batch I	72.9 ± 5.65	105.0 ± 10.4
Batch II	66.2 ± 2.6	95.0 ± 5.0
Batch III	70.9 ± 2.7	
Batch IV	80.0 ± 0	
Batch V	90.0	

[a] Phosphate release experiments were done as described in Methods using *Citrobacter* sp. strain N14 and the phosphomonoesterase (phosphatase) deficient mutant M1, having less than 5% of the activity of the parent strain. Data are means ± standard errors for the independent batches shown. The number of experiments for each batch is shown in parentheses.

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