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Phosphonate utilization by bacteria in the presence of alternative phosphorus sources

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Abstract

Batch and continuous culture experiments were carried out to investigate the effect of orthophosphate and p-nitrophenylphosphate on the utilization of various phosphonates as a P source by bacteria. Detailed tests with methylphosphonate as a model phosphonate and the phosphonate-degrading *Pseudomonas paucimobilis* strain MMM101a revealed that, in contrast with the majority of literature data, the phosphates did not suppress phosphonate utilization. Under conditions of P stress, strain MMM101a simultaneously took up both P-sources, with a preference for the phosphate-P. Study of the kinetic parameters for strain MMM101a, growing on the different P sources revealed similar, rather low, maximum growth rates (ca. 0.15 h^{-1}). However, the affinity for orthophosphate (K_s: 0.17 μM), was more than two orders of magnitude higher than for methylphosphonate (K_s: 66 μM), which might account for the preferential uptake of orthophosphate. Cellular phosphorus yields in continuous cultures varied considerably with the conditions applied. The results suggest that phosphonate degradation can occur also in environments with substantial backgrounds of phosphate.

Abbreviations: D2010 – Dequest 2010, Hydroxyethane diphosphonic acid, D2041 – Dequest 2041, Ethylenediamine tetramethylphosphonic acid, Gly – Glyphosate, N-Phosphonomethyl glycine, HEPES – N-2-Hydroxy-Ethylpiperazine -N' -2-EthaneSulfonic acid, MeP – Methylphosphonate, OD_{650}, OD_{413} – Optical densities respectively at 650 and 413 nm, Pi – Orthophosphate, Pn – Phosphonate-phosphorus, pNPP – para-Nitrophenylphosphate, pNP – paranitrophenol, D – dilution rate of chemostat, day^{-1}

Introduction

Thousands of tons of synthetic phosphonates are used yearly worldwide, mainly as detergent additives and agrochemicals (Egli 1988). The knowledge of phosphonate (bio)degradation is quite fragmentary, particularly with respect to their fate in water, sediment and soil environments. Whereas these compounds generally fail to serve as C or N sources, several laboratory studies report phosphonate utilization by bacteria, when supplied as the sole source of P (Balthazor & Hallas 1986; Cook et al. 1978; Fitzgibbon & Braymer 1988; Pipke & Amrhein 1988a, b; Schowanek & Verstraete 1990; Wackett et al. 1987b, etc.). Indeed, orthophosphate (Pi) has been found to suppress phosphonate utilization (Rosenberg & La Nauze 1967; Daughton et al. 1979; Pipke et al. 1987; Wackett et al.

1987b). As many environments contain relatively elevated concentrations of free Pi and other readily accessible P sources, it is of direct environmental relevance to study phosphonate utilization by bacteria in the presence of alternative P-sources.

The suppression by Pi fits in the theory that phosphonate degradation accompanied by C-P bond cleavage, would both be physiologically (phosphonate uptake and C-P lyase activity) and genetically (C-P lyase synthesis) regulated as a part of the Pho regulon. The gene products of the Pho regulon form a system that particularly accounts for the acquisition of orthophosphate from P-containing compounds at low levels. The system includes for example the high-affinity phosphate starvation transport (PST) system and a non-specific alkaline phosphatase. The Pho regulon is known to be both positively and negatively controlled in *Escherichia coli* (Stock et al. 1989; Wackett et al. 1987a); all operons in the Pho regulon are expressed at low phosphate levels by means of a common transcriptional activator (PhoB) and a histidine kinase (PhoR) system, while high levels of phosphate cause a repression.

Several publications report a diauxic growth pattern in batch tests, when Pi and phosphonates are both present in bacterial growth medium (Rosenberg & La Nauze 1967; Wackett et al. 1987b), indicating a shift from Pi to the phosphonate as the P source. Other studies, however, failed to demonstrate a substantial transition phase in the growth curves (Quinn et al. 1989; Weissenfels M.S. thesis 1987, Bochum University, Federal Republic Germany). The reasons for this difference are unclear so far. In order to assess the fate of phosphonates disposed in environments with parts per million levels of phosphate, we decided to investigate these aspects more closely for some of our phosphonate degrading bacterial strains. In contrast to the above mentioned studies, batch tests were supplemented with continuous culture experiments.

Materials and methods

Reagents and glassware

The formulas, sources and purities of the phosphonates used in this study are listed in Schowanek & Verstraete (1990). pNPP was obtained from Merck. The contamination of pNPP by Pi amounted to approx. 1.5% of the total P. Some spontaneous hydrolysis of pNPP to pNP (BMM medium, 28° C) occurred at the rate of ca. 1% per day. To avoid significant background levels of Pi, the pNPP and phosphonates were filter sterilized, and the media used were maximum four days old. All chemicals were analytical grade. Glassware was cleaned carefully as previously described (Schowanek & Verstraete 1990). Media and stock solutions were prepared with Milli-Q water (Milli-Q system, Waters Associates, Inc.; Resistivity > 18 MOhm · cm).

Media

The defined bacterial growthmedium BMM (Schowanek & Verstraete 1990) was used throughout the tests (pH 7.2). It was supplemented with different P sources in function of the experiments. For the chemostat tests, one drop of silicone antifoam was added per liter.

Cultures of microorganisms

All phosphonate-degrading strains used in this study have been described previously (Schowanek & Verstraete 1990). Experiments have particularly been carried out with the broad spectrum phosphonate-degrading strain encoded MMM101a. This non-motile gram-negative aerobic rod was isolated from hospital activated sludge, and was tentatively identified as a *Pseudomonas paucimobilis* by means of the API tests (API 20NE, 20B, and API-ZYM system, Montalieu Vercieu France) and microscopical examination. Strains MMM101a and MMM412b correspond to subcultures of the natural isolates respectively coded as MMM101 and

MMM412, which are described in Schowanek & Verstraete (1990). Strain MMM101a has been deposited at the Belgian Coordinated Collections of Microorganisms.

Batch and chemostat cultures

Batch experiments were carried out in duplicate under axenic conditions, in 250 ml erlenmeyer flasks incubated aerobically on a rotary shaker (150 rpm, 28° C, darkness). The flasks contained 100 ml BMM medium, to which phosphate-P and/or phosphonate-P was added as the P-source(s) for growth, depending on the experiment. The tests were inoculated with 3 ml of an active inoculum. It is stressed that prior to the batch tests, all strains had been subcultured twice on BMM medium with Pi (300 μM), and not on phosphonates. Eight ml samples were taken at regular intervals, and the OD_{650}, the Pi and Pn concentration were determined as described in Schowanek & Verstraete (1990).

Specific growth rates and generation times in batch test were estimated from logarithmically plotted growth curves. The curves were recorded in duplicate, and the average μ value was calculated.

Chemostat experiments were carried out with a 280 ml fermentor, which was stirred at 180 rpm and aerated at a flow rate of ca. 30 ml air $\cdot$ min^{-1}. All experiments were performed at 24° C. Air inlets and outlets were kept sterile by means of 0.22 μm Millipore filters plus a glass-fiber prefilter. Growth- and P-utilization kinetics in continuous cultures were determined according to Bailey & Ollis (1986) from 1/S versus 1/D plots, obtained by varying the D at a constant influent-P concentration. The biomass density in the reactor was estimated routinely by means of the OD_{650}, as a rapid indirect measurement. The relation between the OD_{650} of the culture, and the suspended solids content (g SS dry weight.$liter^{-1}$) for strain MMM101a was the following: $OD_{650} = 2.040\,SS + 0.047$ ($r^2 = 0.94$). This relation was linear up to an OD_{650} of ca. 1.6. Samples with a OD_{650} above 1.0 were diluted 1/2 or 1/3 (v/v) with fresh BMM medium for the final reading.

The yield factor on phosphorus, i.e. the biomass produced per unit P taken up, was calculated as follows: $Y_P = X/P_{consumed}$ (X = in-reactor biomass as OD_{650} or as mg SS $\cdot$ l^{-1}; $P_{consumed} = P_{feed} - P_{reactor}$, in μg-atoms P $\cdot$ l^{-1} or as mg P $\cdot$ l^{-1}).

Analytical methods

– Determination of phosphorus-containing compounds was performed on an Auto-analyser system (AAII system, Technicon Inc.), as described in Schowanek & Verstraete (1990). In experiments with a mixture of a phosphonate (MeP) and a phosphate (pNPP), it was not possible to follow the utilization of both organic phosphorus compounds separately, and only total phosphorus removal was followed. Concentrations of P-containing compounds were expressed as μM, or as μg-atoms P $\cdot$ l^{-1} (1 μM corresponds with 1 μg-atom P $\cdot$ l^{-1} for monophosphates or monophosphonates).

– Total P in biomass (mg P/mg SS): 5 ml culture was centrifuged (20 min × 7000 g), washed with physiological solution, and recentrifuged. The pellet was resuspended in 4 ml ammoniumpersulfate solution (400 g/l) and digested in an autoclave (35 min at 121° C). This digestion was repeated with an extra 4 ml ammonium persulfate solution, after which a completely clear digest was obtained. Pi in the sample was measured on the auto-analyzer. The SS content of the culture was determined on a separate sample.

– Phosphatase activity was determined with pNPP as a substrate, according to the principle outlined by Kersters & De Ley (1971). *Pseudomonas paucimobilis* strain MMM101a shows both acid and alkaline phosphatase activity. Alkaline phosphatase activity in the culture supernatant was taken as an indicator for the expression of the Pho regulon and was measured as follows: 5 ml centrifuged sample supernatant (20 min × 7,000 g) is mixed with 5 ml Tris.HCl buffer (pH 9.0, 40 mM) to which 100 μM pNPP is added. This mixture is incubated in the dark at 28° C, and the change in OD_{413} is measured after exactly one hour. The specific alkaline phosphatase activity of the culture is calculated as the ratio of the Δ OD_{413} (in 1 hour)

over the suspended solids content of the culture ($gSS \cdot l^{-1}$) that had produced the supernatant.

– Optical density measurements: OD measurements were made on a Shimadzu UV190 type spectrophotometer.

Results and discussion

Batch culture experiments on phosphonate degradation in the presence of phosphates

Several phosphonate-degrading bacteria from our laboratory culture collection were selected for research on the effect of alternative phosphorus sources on phosphonate degradation, i.e., *Arthrobacter* sp. strain GLP-1, *Pseudomonas* sp. strain PG2982, and the polyphosphonic acid-degrading isolates coded MMM101a and MMM412b. Strain MMM101a had been tentatively identified as a *Pseudomonas paucimobilis,* while strain MMM412b had not further been examined. The phosphonate-degrading capacities of the test strains have previously been described (Pipke et al. 1987; Pipke & Amrhein 1988a, b; Fitzgibbon & Braymer 1988; Schowanek & Verstraete 1990). Preliminary tests revealed that, besides the Dequest polyphosphonates, our isolates MMM101a and MMM412b were also able to grow on various monophosphonates like MeP and glyphosate, as their sole source of P. Methane formation was observed for all strains with MeP (not shown).

In a first series of experiments, the strains were grown in batch in BMM medium containing ca. 150 μg-atoms $\cdot l^{-1}$ of Pn (= 5 to 18 mg phosphonate $\cdot l^{-1}$, depending on the type of phosphonate) plus a background of ca. 50 μg-atoms $\cdot l^{-1}$ of Pi ($\simeq 5$ mg $\cdot l^{-1}$ PO_4^{3-}). The latter concentration corresponds to a normal concentration of Pi in domestic sewage (Nesbitt 1973). The phosphonate concentration was taken in the same order of magnitude for analytical reasons, although the predicted environmental concentration (PEC) of phosphonates in raw sewage is at least 10 times less. The tests were confined to the monophosphonates MeP and Gly, and the polyphosphonates D2010 and D2041. All experiments were done in duplicate.

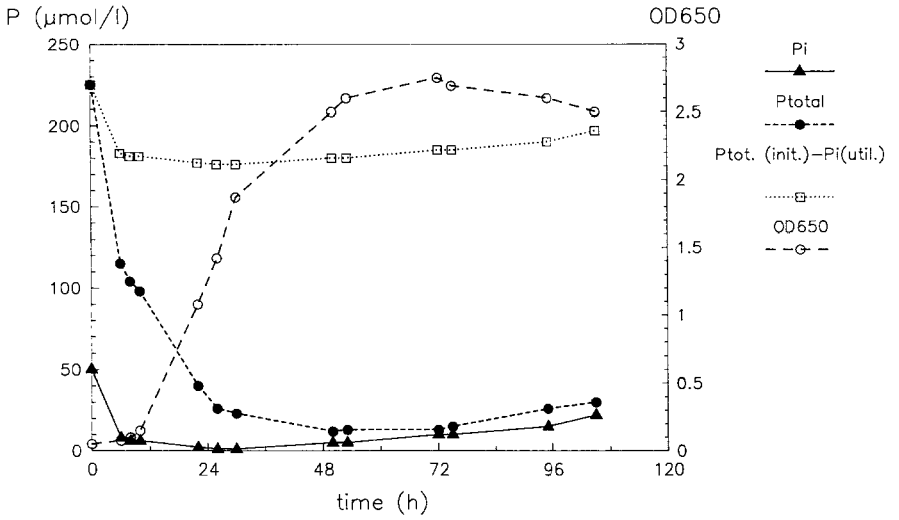
The growth curves, and the time-courses for Pi and Pn (Pn = Ptotal – Pi) were recorded for each combination of phosphonate and bacterium. *Pseudomonas* sp. PG2982 was not tested against D2010 and D2041, as it does not utilize these phosphonates. Somewhat to our surprise, no diauxic growth patterns were observed in these experiments: at the moment of complete Pi exhaustion, no significant lag-phase in the growth curves was noticeable. An example of an entire batch test with strain MMM101a growing on MeP and Pi is shown in Fig. 1a. Similar results were found in the other tests (a small initial growth lag, in the order of 24 h, was noted for *Arthrobacter* sp. strain GLP-1 and strain MMM101a in the presence of D2010, but not with the other phosphonates; not shown). The evolution of the Pi and the Pn concentration during the first hours of growth was investigated in more detail in a separate experiment (Fig. 1b), carried out under identical conditions as for Fig. 1a (BMM medium, 28° C, Pi + MeP, strain MMM101a). The latter test indicated that simultaneous uptake of Pn and Pi can take place under these circumstances. Furthermore, the cells removed rapidly all Pi and a large fraction of the Pn before full exponential growth occurred. This indicates that large reserves of P can be stored by the cells (it should be noted here that the inoculum was characterized by a state of moderate P deficiency when added).

These observations led us to investigate the effects of orthophosphate-P as well as organic phosphates on phosphonate utilization in more detail. Further testing was restricted to strain MMM101a, and MeP as a model phosphonate. MeP was selected because it is non-toxic and does not show any spontaneous degradation. It yields only methane and Pi as metabolites, according to the reaction: $CH_3 - PO_3H_2 + H_2O \rightarrow CH_4 + Pi$.

Specific growth rates on different P-sources

The specific growth rates for strain MMM101a grown with 300 μg-atoms phosphate-P or phosphonate-P $liter^{-1}$ were determined on carefully recorded batch growth curves. The μ-values recorded for Pi, MeP, pNPP and D2010 amounted to 0.15,

a



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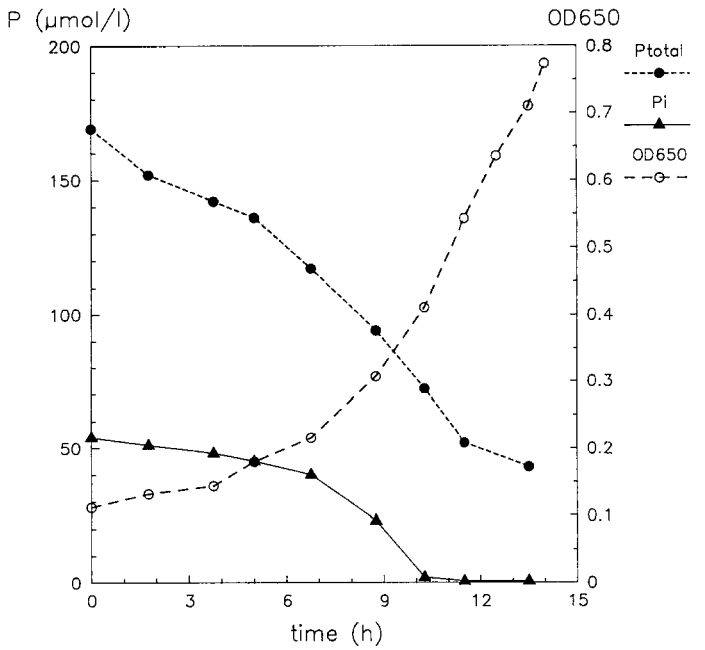

Fig. 1. (*a*) Growth curve, and Pi and MeP removal by strain MMM101a grown in BMM medium (28° C) for the complete batch culture. Some Pi was released by the cells in the stationary phase. The upper curve (squares) represents the Ptotal initial ($\simeq 225\ \mu$g-atoms P · liter^{-1}) minus the Pi taken up, in function of time. This illustrates more clearly the respective fractions of Pi and MeP consumed. (*b*) Evolution of Pi, Pn and biomass levels during the first 15 hours of incubation (results from a separate experiment under identical conditions).

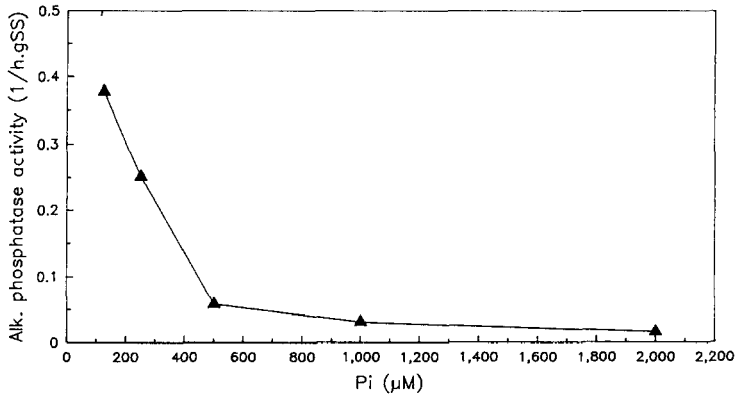

Fig. 2. Expression of alkaline phosphatase activity (Δ OD_{413}/h · gSS) by strain MMM101a grown in BMM medium (28° C, dark) in function of the Pi concentration. 100 μM MeP was present as an organic P source. Measurements were made after 48 h of growth.

0.12, 0.13 and 0.075 h^{-1} (generation times 4.62, 5.78, 5.33 and 9.24 h), respectively. It was noted that MMM101a is a rather slow-growing organism, with clearly reduced growth rates and a 24 h growth lag on the diphosphonate D2010 (not shown).

The previous tests indicated that strain MMM 101a, as it grew well on 300 μM pNPP as a P-source, expressed the Pho regulon (including alkaline phosphatase) under these conditions. Phosphatase activity was also revealed by the yellow coloration (pNP formation) of the culture. In the absence of an *in-vitro* test for C-P lyase, the expression of the Pho regulon was studied by means of the alkaline phosphatase activity. To check whether the Pho regulon in strain MMM101a is controlled in a way similar to *E. coli* (Rosenberg et al. 1977) and *Pseudomonas aeruginosa* (Lacoste et al. 1975), the level of alkaline phosphatase activity was measured in function of the Pi present in the medium. Strain MMM101a was cultured in medium with a background concentration of an organic P-source (in casu MeP, 100 μM) and increasing concentrations of Pi. The enzyme activities measured in the supernatant at the different Pi levels were calculated per unit biomass present in the original cultures.

The results of this test are shown in Fig. 2. Above 500 μM Pi, a clear suppression of phosphatase activity is apparent. This level corresponds well with the results obtained by Rosenberg et al. (1977) with *E. coli,* who reported a suppression of the PST system (and the Pho regulon) above 1 mM Pi. A configuration of phosphate transport with a constitutive, rather insensitive Pi uptake system (type PIT), and an inducible high-affinity system (type PST) has been reported for an increasing number of microorganisms (Barchietto et al. 1989). The pattern of phosphatase expression in function of the Pi concentration for strain MMM101a can be an indication of a comparable organization.

Considering that at low levels of Pi (e.g. 50 μM) the Pho regulon will be fully expressed in strain MMM101a, the simultaneous utilization of Pi (via the PST system) and phosphonates (via an uptake system + C-P lyase enzyme) would also seem theoretically possible.

It should be noted that no C-P lyase activity in vitro could be detected with supernatants of cultures of strain MMM101a grown on MeP and D2010. Both cultures showed high levels of alkaline phosphatase activity, however. This illustrates once more that both P sources require specific enzymes, and that C-P lyase is not extracellular.

Continuous culture experiments with mixtures of P-sources

Further experiments were carried out with continuous cultures of strain MMM101a. This allowed to simulate a situation where a phosphonate as well as a phosphate are continuously supplied to the cells as P-sources. The experiments were carried out

under conditions of overall P-limitation as observed in batch cultures, i.e. Ptotal in the feed $< 300\,\mu$g-atoms.l^{-1} (Schowanek & Verstraete 1990).

First, an estimation of the kinetic parameters μ_{max} and K_s for strain MMM101a grown on Pi and MeP was obtained by measuring their in-reactor concentration (= effluent concentration) at various dilution rates. The influent concentration was kept constant at 50 μM Pi or MeP. The results are shown in Table 1. It can be concluded that strain MMM101a is characterized by a moderate affinity for MeP, while it has a markedly higher affinity for Pi. The K_s value for Pi is very close to the value of 0.16 μM reported by Rosenberg et al. (1977) for the PST system in *E. coli*. Pipke et al. (1987, *Arthrobacter* sp. strain GLP-1) and Fitzgibbon & Braymer (1988, *Pseudomonas* sp. strain PG2982) reported K_s values for glyphosate uptake of 125 and 23 μM, respectively. A higher affinity constant of 0.1 μM for 2-aminoethylphosphonate uptake was found in *Bacillus cereus,* while the affinity of the phosphonatase enzyme itself amounted to 40 μM (La Nauze et al. 1970). The maximum growth rates of strain MMM101a are similar to those reported by Quinn et al. (1989) for *Pseudomonas* sp. 4ASW; 0.11 (MeP, 0.5 mM) and 0.23 (Pi, 0.5 mM) h^{-1}.

Subsequently, strain MMM101a was tested with a mixture of Pi and MeP as P sources for growth (Fig. 3). Initially, medium with MeP only (50 μM) was fed to the reactor. The behaviour of the reactor parameters OD_{650}, and the concentration of Pi and MeP were closely monitored. First, different dilution rates were applied, mainly to check the growth levels at the respective D values. At a moderate D of 0.6 day^{-1}, the reactor was allowed to equilibrate ($OD_{650} \simeq 0.520$) and subsequently an extra P source under the form of 20 μM Pi was added to the influent. After a temporary dip of the OD_{650}, the reactor equilibrated at a higher OD_{650} level ($\simeq 0.750$). The evolution of Pi and MeP indicated that both phosphorus compounds disappeared from the medium. The addition of Pi was not registered in the effluent, which was characterized by constant and low Pi levels. At 380 h, MeP was omitted from the influent, which resulted in a gradual decrease of the OD_{650} level to 0.220. The results clearly show that MeP contributes to the cell growth, even in the presence of Pi.

Table 1. Kinetic parameters for strain MMM101a grown on Pi (50 μM) or MeP (50 μM) in BMM medium (pH 7.2, 24° C).

P-source	r^{2*}	Parameter	
		K_s (μM)	μ_{max} (h^{-1})
Pi	0.96	0.17	0.19
MeP	0.97	66	0.15

* r^2: linear regression coefficient of the 1/D against 1/S plot.

In the following run, the Pi concentration and the dilution rate were kept constant at 20 μM and 0.6 day^{-1} respectively over the entire experiment, but the MeP concentration was increased in steps from 0 to 280 μM. The biomass level in the reactor, and the Pi and MeP concentration were monitored. The time-courses are shown in Fig. 4. Each time the culture was fed with an influent with increased MeP levels, the in-reactor biomass concentration increased. Again, the Pi was taken up completely, while MeP was removed to a very large extent, even at high influent-P concentrations. It was noted that an increase of the P content of the feed tended to cause a temporary depression of growth, and a decreased phosphonate removal. The underlying mechanism of this phenomenon is not clear to us so far, but it can not be attributed to induction, as the culture already utilized MeP. Once more, these data illustrate the potential of strain MMM101a to grow on Pi and phosphonates simultaneously.

Finally, a series of analogous experiments were performed with combinations of the organic phosphate pNPP and one or more phosphonates. For example, a mixture of the organic phosphate pNPP (50 μM) and the phosphonate MeP (50 μM) was fed to the reactor. The D was set at 0.6 day^{-1}. The results obtained were analogous to those with Pi and MeP. A simultaneous utilization of both organic P sources was found, as the Ptotal in the reactor remained below 15% of the influent concentration. In contrast to the tests with Pi (with or without another P source), some background Pi (2–4 μM) was constantly present in the reactor (not shown).

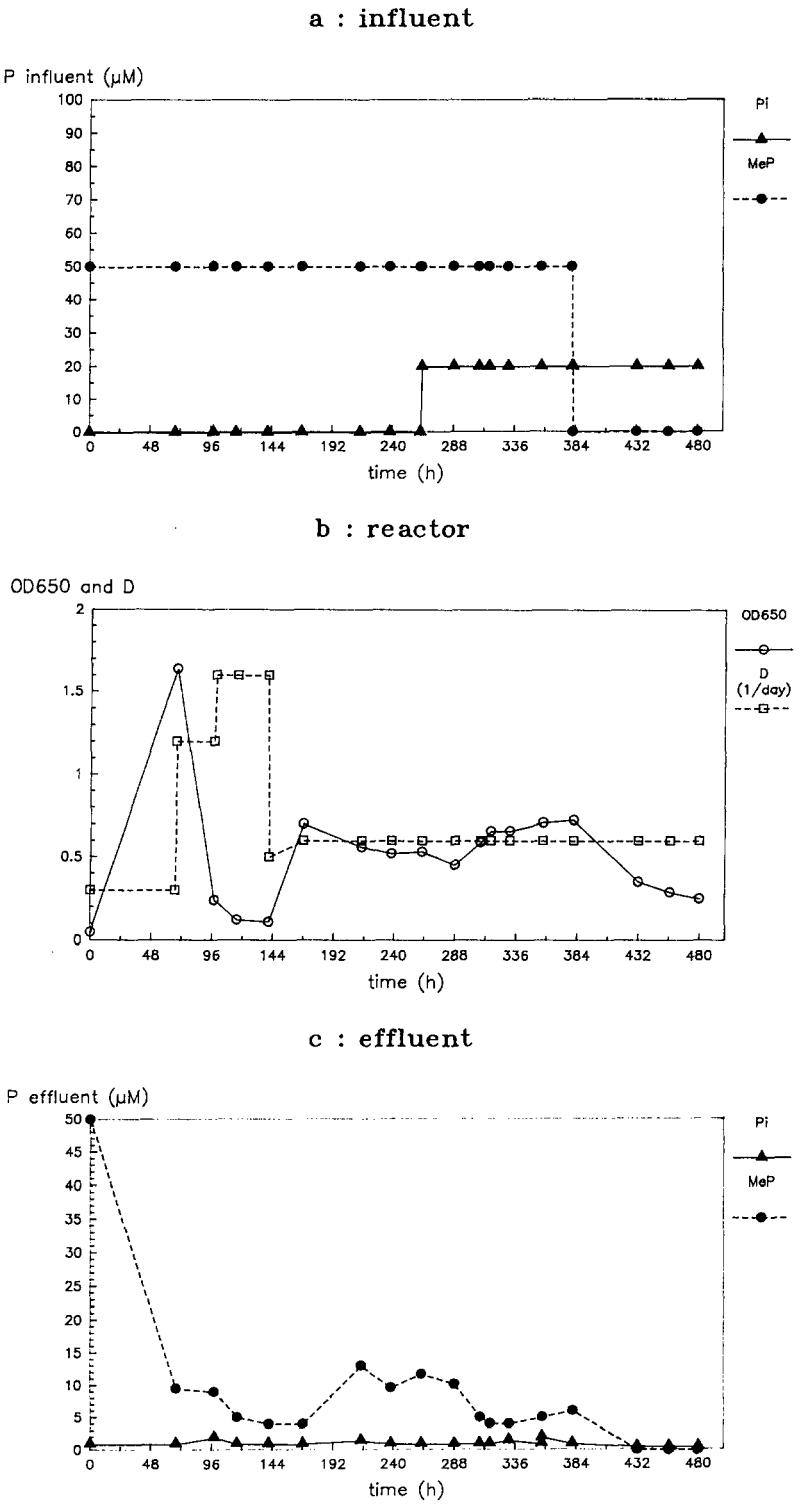

Fig. 3. Chemostat experiment with strain MMM101a and a mixture of P sources (BMM, 24° C): evolution of the biomass, and Pi and MeP levels in the effluent in function of the dilution rate and the phosphorus source.

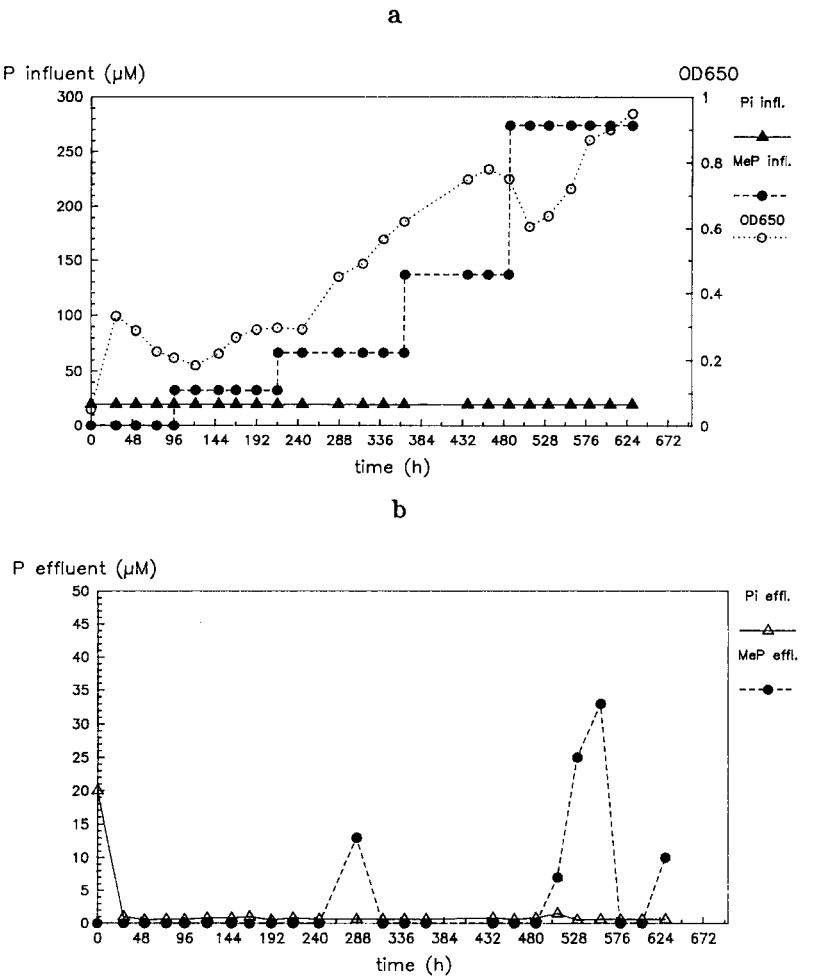

Fig. 4. Chemostat experiment with strain MMM101a (BMM medium, 24° C) supplied with increasing MeP concentrations (0–280 μM), in the presence of a constant Pi background level (20 μM) and at a constant dilution rate (0.6 day^{-1}). a: influent and reactor. b: effluent.

A puzzling aspect of the chemostat experiments was the cellular phosphorus yield (Y_p), i.e. the biomass produced per unit of P consumed ($\Delta OD_{650}/\mu$g-atom P), for the different experiments. In batch tests, the Y_p for strain MMM101a was found to be independent of the P source and relatively constant, this is, 0.013 to 0.020 OD_{650} units of biomass formed per μg-atom P consumed (cf. for instance Fig. 1a; values calculated for the max. OD_{650} in the early stationary phase). Expressed as mg SS/mg P this amounts from 198 to 309, or a P content on cell dry weight of 0.32 to 0.50%. The calculated Y_p in continuous culture, however, varied according to the conditions applied. At a moderate growth rate of 0.6 day^{-1} on MeP (Fig. 3, 260 h) the Y_p amounted 0.0141 (corresponding to a calculated P content of 0.46%), which is a figure comparable to the batch tests. The P content of the biomass at that moment measured by digestion reached 0.97 ± 0.04%, however. This indicates that the cells store P under these conditions rather than making full use of it for growth. There was a tendency for an increased P content in the biomass of MMM101a grown on MeP (50 μM) at higher dilution rates (= younger cells; data obtained in the tests for the determination of the kinetic parameters): a value of 2.85 ± 0.23% P was noted at D = 1.2 day^{-1}, a situation where the in-reactor biomass was low (OD_{650} ca. 0.055), but the MeP removal still ca. 70%.

These aspects were not further investigated as they relate only indirectly to phosphonate biodegradation. It can be added however that large variations in Y_p in continuous cultures have been reported in literature (Tempest 1970), pointing at the potential of the cells for so called luxury phosphorus uptake, and also that high levels of ATP and RNA have been reported in young bacterial biomass (Herbert et al. 1971; Verstraeten et al. 1983).

Conclusions

- Simultaneous phosphate and phosphonate utilization by bacterial strains was demonstrated. The phosphonates clearly contributed to cell growth, even in the presence of phosphates. This suggests that the presence of phosphates in environmental samples does not necessarily exclude phosphonate degradation. The effect of phosphates at high levels (> 1 mM) requires further investigation.

- Phosphorus uptake in the phosphonate degrading strain *Pseudomonas paucimobilis* strain MMM101a might be organized in a similar way as in *E. coli* K12 (Wackett et al. 1987a), at least with respect to the presence of a dual uptake system. Some indications for a high affinity phosphate uptake system in strain MMM101a, with a K_s value of 0.17 μM and which is suppressed above 500 μM Pi, were found. No diauxic growth pattern was observed with a mixtures of Pi and Pn.

- Cultures grown on phosphonates expressed high levels of alkaline phosphatase activity. No extracellular C-P lyase activity was detected.

- In all experiments, Pi was the prefered P-source of the bacteria. This is attributed to the significantly higher affinity of the cells for Pi, compared to phosphonates, rather than to repression of Pn utilization.

- The Y_p values and P content of the cells varied considerably in the continuous cultures according to the growth conditions imposed. The amount of P stored in the cells was particularly high at high dilution rates.

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