Phosphatase production and activity in *Citrobacter freundii* and a naturally occurring, heavy-metal-accumulating *Citrobacter* sp.

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The ability of a naturally occurring *Citrobacter* sp. to accumulate cadmium has been attributed to cellular precipitation of CdHPO_4 , utilizing HPO_4^{2-} liberated via the activity of an overproduced, Cd-resistant acid-type phosphatase. Phosphatase production and heavy metal accumulation by batch cultures of this strain (N14) and a phosphatase-deficient mutant were compared with two reference strains of *Citrobacter freundii*. Only strain N14 expressed a high level of acid phosphatase and accumulated lanthanum and uranyl ion enzymically. Acid phosphatase is regulated via carbon-starvation; although the *C. freundii* strains overexpressed phosphatase activity in carbon-limiting continuous culture, this was approximately 20-fold less than the activity of strain N14 grown similarly. *Citrobacter* strain N14 was originally isolated from a metal-contaminated soil environment; phosphatase overproduction and metal accumulation were postulated as a detoxification mechanism. However, application of Cd-stress, and enrichment for Cd-resistant *C. freundii* ('training'), reduced the phosphatase activity of this organism by about 50% as compared to Cd-unstressed cultures. The acid phosphatase of *C. freundii* and *Citrobacter* N14 had a similar pattern of resistance to some diagnostic reagents. The enzyme of the latter is similar to the PhoN acid phosphatase of *Salmonella typhimurium* described by other workers; the results are discussed with respect to the known phosphatases of the enterobacteria.

Keywords: *Citrobacter*, phosphatase, heavy metals, cadmium resistance

INTRODUCTION

Strains of *Citrobacter* spp. isolated from lead-contaminated soil (Aickin & Dean, 1979; Macaskie & Dean, 1982, 1984) accumulated heavy metals when resuspended in metal-supplemented solution (Aickin & Dean, 1979; Macaskie & Dean, 1984). Metal uptake is mediated via the activity of an overproduced acid-type phosphatase, induced during pre-growth, that functions also in non-growing, resuspended cells and in immobilized cells to liberate HPO_4^{2-} from appropriate organic phosphate 'donor' molecules (substrates) with precipitation of heavy metals as (e.g.) PbHPO_4 (Aickin *et al.*, 1979), CdHPO_4 (Macaskie *et al.*, 1987) or UO_2HPO_4 (Macaskie *et al.*, 1992) at the cell surface. The lead-accumulating and cadmium-accumulating *Citrobacter* spp. were different isolates, as suggested by their respective responses to a range of standard biochemical tests (strain identification reports, National Collection of Industrial and Marine

Bacteria, 1978, 1981) and in the inability of the lead-accumulating strain to accumulate Cd^{2+} (R. M. Aickin, L. E. Macaskie & A. C. R. Dean, unpublished). In contrast, the Cd-accumulating strain was non-specific with respect to the metals accumulated; these included Cd^{2+} , Pb^{2+} , Cu^{2+} , Sr^{2+} , UO_2^{2+} (Macaskie, 1990), Mn^{2+} (Clark *et al.*, 1992, 1993), La^{3+} , Am^{3+} , Th^{4+} and Pu^{4+} (Macaskie *et al.*, 1994a), and the potential of this strain in the bio-remediation of metal- and radionuclide-contaminated wastes has been suggested (Macaskie *et al.*, 1994a).

Since two separate *Citrobacter* strains were isolated from the metal-polluted site it was of interest to establish whether the phosphatase overproduction and metal-accumulative properties are unique to these strains (possibly selected via metal stress) or are a more general feature of the genus *Citrobacter*. Silver uptake using a *Citrobacter* sp. was identical from substrate-supplemented and substrate-free suspensions, discounting a major role

for phosphatase in this case (Goddard & Bull, 1989), while other studies have demonstrated *Citrobacter*-mediated accumulation of uranyl ion (Watson & Ellwood, 1987; Bahaj *et al.*, 1989); it was not clear to what extent phosphatase activity was contributory.

One hypothesis suggests that prolonged exposure to heavy metals can elicit phosphatase overproduction as a detoxification mechanism, with precipitation of metals away from sensitive cellular sites. Additionally, the Cd resistance of the enzyme (Hambling *et al.*, 1987) suggests that the enzyme itself may have evolved a metal tolerance. These predictions were tested in the present study, which also sought to investigate other possible differences in the phosphatase enzyme(s) of the naturally occurring isolate as compared to standard strains of *C. freundii* obtained from a culture collection and grown in batch and continuous culture.

METHODS

Micro-organisms. *Citrobacter* sp. strain N14 was isolated from a site contaminated with lead as described previously (Macaskie & Dean, 1982) and was identified to genus level by staff of the National Collections of Industrial and Marine Bacteria (NCIMB; Torry Research Station, Aberdeen, UK). Cultures were routinely checked using the API 20E system of classification (API Products), and differed from the normal *Citrobacter* response to the lysine decarboxylase test; the high diversity of soil *Citrobacters* has been noted by Farmer (1981). Screening for plasmids was done by Dr L. Diels (VITO, Mol, Belgium); none were found. The phosphatase-deficient mutant lp4a was as described previously (Macaskie *et al.*, 1994a). The phosphatase deficiency of the mutant was confirmed by assay and by immunogold staining (Jeong, 1992). The type strains of *Citrobacter*, *Citrobacter freundii* NCIMB 11490 and *Citrobacter intermedium* NCIMB 7271, were obtained from the NCIMB. *C. intermedium* NCIMB 7271 has since been redesignated as *C. freundii* (NCIMB Catalogue, 1990).

Growth of the organisms. The *Citrobacter* strains were maintained aerobically at 30 °C by daily subculture in minimal medium of the following composition (g l⁻¹): Tris-base, 12.0; (NH₄)₂SO₄, 0.96; glycerol 2-phosphate (disodium salt hydrate, 5.5H₂O), 0.67; KCl, 0.62; MgSO₄·7H₂O, 0.063; FeSO₄·7H₂O, 0.00032; glycerol, 1.5 or as otherwise specified; pH adjusted to 7.0 with 2 M HCl. The $\mu_{\max}$ (maximum specific growth rate) was determined in fully adapted cultures in this medium. 'Training' to Cd²⁺ resistance was done by sequential batch subculture in minimal medium supplemented with progressively increasing concentrations of Cd²⁺ to a final Cd²⁺ concentration of 100 µg ml⁻¹ (0.89 mM). The maximum specific growth rate in Cd-medium was determined after 10 subcultures at 50 µg Cd²⁺ ml⁻¹ and then 10 subcultures at 100 µg ml⁻¹ via progressive steps of 65 µg ml⁻¹ (five subcultures), 80 µg ml⁻¹ (nine subcultures) and 90 µg ml⁻¹ (two subcultures).

Continuous culture. Carbon-limiting and phosphate-limiting media were as above, except that the concentrations of glycerol and glycerol 2-phosphate were reduced to 0.6 and 0.066 g l⁻¹ respectively; in the latter medium the concentration of glycerol was increased to 3 g l⁻¹. Polypropylene glycol antifoam was incorporated at 1 ml per 10 l of medium. The cultures were maintained at 30 °C in a New Brunswick 'Bioflo' apparatus (agitation 400 r.p.m., air flow 0.5 l min⁻¹) at a dilution rate corresponding to $D = 0.5 \mu_{\max}$. This was equivalent to $D =$

0.25 h⁻¹ for *Citrobacter* N14, $D = 0.28 \text{ h}^{-1}$ for *C. freundii* NCIMB 11490 and $D = 0.32 \text{ h}^{-1}$ for *C. freundii* NCIMB 7271. The pH of phosphate-limited cultures tended to fall; this was held at pH 7 using an automated feed of NaOH (2 M). Steady states were reached in the chemostats after 24 generations of growth, after which the chemostat outflows were collected at 0 °C.

Harvesting and storage of the cells. The cells were harvested from batch cultures in the mid-exponential phase (OD₆₀₀ 0.4 approx.), or from the chemostat outflows, by centrifugation, washed in isotonic saline (8.5 g NaCl l⁻¹) and stored at 4 °C, as described previously (Hambling *et al.*, 1987).

Assay of phosphatase activity. Phosphatase activity was estimated in triplicate samples by the liberation of *p*-nitrophenol from *p*-nitrophenyl phosphate (disodium salt; BDH) as described by Bolton & Dean (1972). Samples (1 ml) of concentrated bacterial suspension (1.2–2.4 mg bacterial dry weight ml⁻¹ in isotonic saline) were diluted to 10 ml in appropriate buffer (below). Magnesium chloride (0.1 ml of 0.15 mM MgCl₂·6H₂O) was added and the mixture was pre-equilibrated at 30 °C for 15 min prior to initiation of the reaction by the addition of substrate (45.6 mM *p*-nitrophenyl disodium orthophosphate: 2 ml). Timed samples (2 ml) were quenched in 4 ml 0.2 M NaOH and the liberated *p*-nitrophenol was estimated spectrophotometrically at 405 nm versus a suitable *p*-nitrophenol standard. Phosphatase specific activity (unit) is defined as nmol *p*-nitrophenol liberated min⁻¹ (mg bacterial protein)⁻¹, with bacterial protein determined by the Lowry method. Experiments used cells withdrawn from two or three independent chemostats for each strain. In the latter case data are expressed as means $\pm$ SEM. Where two chemostats were used the data (three determinations from each chemostat) were pooled to give the data from two experiments. The mean $\pm$ SEM for the six samples was generally within 10–15%.

To test the effect of a heavy metal, activator or inhibitor, the cells were pre-equilibrated for 1.5 h in the reaction mixture at pH 5 (MES buffer), pH 7 (MOPS buffer) or pH 8.5 (Tricine buffer; see below) containing the agent under test prior to the addition of *p*-nitrophenyl phosphate. Metal solutions were freshly prepared prior to their use, and controls established that no metal precipitation occurred in cell-free media. Where the quenched phosphatase reaction yielded a metal phosphate or hydroxide precipitate the solid material was allowed to settle out (1–2 h) prior to estimation of *p*-nitrophenol in the supernatant.

Buffer systems for phosphatase assay. Determination of pH-activity profiles (pH 3–10) necessitated the use of several buffers (all 200 mM), as follows: pH 3–4, citric acid/trisodium citrate; pH 4–6, acetic acid/sodium acetate; pH 5, MES/NaOH; pH 6–9, Tris/HCl; pH 7, MOPS/NaOH; pH 8.5, Tricine/NaOH; pH 9–10, sodium carbonate/sodium hydrogen carbonate. The use of two buffers at overlap pH values allowed for correction of effects of the buffer on enzyme activity. In general, little difference was observed, except that phosphatase activity was stimulated by 3.8 $\pm$ 0.2-fold at pH 9 in Tris buffer as compared to carbonate buffer under the same conditions (mean $\pm$ SE, three experiments). Cell permeabilization was not required to visualize phosphatase activity (Hambling *et al.*, 1987) but additional checks were made as appropriate by treatment with toluene/ethanol (1:9, v/v) (Carter & Dean, 1976).

Heavy metal accumulation by *Citrobacter* spp. Batch-grown cells (metal-unsupplemented) were harvested in the mid-exponential phase and challenged with either uranyl nitrate [UO₂(NO₃)₂·6H₂O] or lanthanum nitrate [La(NO₃)₃·6H₂O] (1 mM) in a carrier solution of 2 mM citrate/20 mM MOPS

buffer, pH 7, supplemented with 5 mM glycerol 2-phosphate, as described previously (Macaskie *et al.*, 1994b). The residual metal remaining in the supernatant following removal of the cells by centrifugation was assayed after 6 h and 24 h using arsenazo III, with metal uptake expressed as a percentage of the initial metal removed (Macaskie *et al.*, 1994b).

RESULTS AND DISCUSSION

Phosphatase activities of batch-grown cells, and heavy metal bioaccumulation

Cultures of *C. freundii* NCIMB 11490 and NCIMB 7271, together with *Citrobacter* sp. N14 and the phosphatase-deficient mutant lp4a were harvested and challenged with uranyl ion (UO_2^{2+}) or lanthanum (La^{3+}) as described previously (Macaskie *et al.*, 1994b). The high phosphatase activity for two specimen batches of strain N14, of 596 and 550 units, correlated with the removal of 97.4 and 98.0% of the UO_2^{2+} , respectively, after 24 h and complete removal of La^{3+} after only 6 h. The activity of batch-grown cells was intrinsically variable; the mean $\pm$ SEM was determined at 327 ± 3 units in initial studies (Hambling *et al.*, 1987) and was 236 ± 19 units (Macaskie *et al.*, 1988) 302 ± 165 units (Butler *et al.*, 1991), and 442 ± 168 units (Macaskie *et al.*, 1994b) in later investigations.

In contrast to strain N14, UO_2^{2+} was not removed by the *C. freundii* strains (the phosphatase specific activity was 3.92 ± 2.38 units and 4.08 ± 1.76 units for strains NCIMB 11490 and NCIMB 7271; means $\pm$ SEM from 9 and 11 experiments, respectively), or by the phosphatase-deficient mutant lp4a derived from strain N14. However, the *C. freundii* strains removed 100% of the La^{3+} after 24 h. This was probably attributable to nonspecific precipitation of La upon the cells or within the periplasmic space with time; an identical result was obtained using the phosphatase-deficient mutant lp4a, which indicated non-specific bioaccumulation mechanisms. A previous study has demonstrated the high capacity of the periplasmic space and surface layers of the related *Escherichia coli* for deposited lanthanide ions (Bayer & Bayer, 1991). Over a shorter timescale (6 h), La^{3+} was completely removed by strain N14 only; none was removed by the two *C. freundii* strains or the phosphatase-deficient mutant. These results, together with a previous demonstration of the removal of heavy metals by strain N14 (but not by the phosphatase-deficient mutant: Macaskie *et al.*, 1994a), confirmed the participation of the phosphatase in heavy metal bio-precipitation.

Studies on metal bioaccumulation were done at neutral pH, since previous work has shown that metal phosphates are more soluble at acidic pH; here the correlation (Macaskie *et al.*, 1994a) between phosphatase activity and metal desolubilization does not apply (Tolley *et al.*, 1995). For a more realistic evaluation of acid phosphatase activity *C. freundii* was also assayed at pH 5. The phosphatase specific activities were increased to 6.4 ± 0.7 units and 7.1 ± 0.6 units for strains NCIMB 11490 and NCIMB 7271, respectively (means $\pm$ SEM; eight experiments), but remained substantially lower than that of strain N14 [the phosphatase activity of batch-grown cells of this strain

(> 200 units; see above) was similar at pH 5 and pH 7; Tolley *et al.*, 1995].

A previous study concluded that the phosphatase specific activity of batch-grown cells of strain N14 increased approximately threefold during exponential growth (Butler *et al.*, 1991). An increase was also seen if the cells were transferred to anaerobic conditions, even though further growth did not occur (Hallett *et al.*, 1991). A corresponding increase was not observed with the *C. freundii* strains following a shift to anaerobiosis (not shown). Phosphatase activity was also monitored in batch cultures harvested during growth. In contrast to strain N14 (above) the increase in phosphatase activity was small for both *C. freundii* strains. The activity of late-exponential-phase cells ($\text{OD}_{600} = > 0.8$) was increased as compared to mid-exponential-phase cells by 2.3 ± 0.8 -fold and 1.8 ± 0.4 -fold at pH 7 and 5, respectively, for strain NCIMB 7271 and 1.3 ± 0.2 -fold and 1.2 ± 0.2 -fold at pH 7 and 5, respectively, for strain NCIMB 11490 (means $\pm$ SEM; six experiments).

These preliminary tests showed little similarity between the phosphatase activity of *Citrobacter* N14 and the *C. freundii* strains. The production of alkaline phosphatase, regulated via phosphate starvation (Wanner, 1993; see below), is well documented, but acid phosphatase production, regulated by carbon starvation in addition to various other stresses such as pH and anaerobiosis (see below), is less well understood. If it is argued that the metal-stress conditions in the original environment may have selected for constitutive phosphatase expression in strain N14 as a metal detoxification mechanism (see Introduction), growth in non-limiting batch cultures might not be expected to promote the expression of either phosphatase by the *C. freundii* strains. Against this, however, is the recent observation that *E. coli*, grown on carbon sources other than glucose, expresses acid phosphatase activity during batch growth (Rossolini *et al.*, 1994). Subsequent experiments used continuous culture to compare phosphatase activities of the strains under steady-state conditions of nutrient limitation in order to express the activity of the phosphatases (where present) maximally.

Alkaline phosphatase activities of *Citrobacter* spp. grown under phosphate limitation

The production and activity of alkaline phosphatase is regulated via the *pho* regulon (Wanner, 1993) and, accordingly, the phosphatase activity of *Citrobacter* sp. N14 and *C. freundii* NCIMB 11490 was maximal at pH 8–8.5 in cells withdrawn from phosphate-limiting continuous cultures (Fig. 1). The specific activity of the two strains was similar, at 200 and 251 units for strains N14 and NCIMB 11490, respectively; this is shown as 100% for both strains (Fig. 1) for comparison. In accordance with earlier results using strain N14 (Hambling *et al.*, 1987) toluenization of *C. freundii* NCIMB 11490 did not yield additional phosphatase activity at pH 8.5. It was concluded that alkaline phosphatase production by the two *Citrobacter* strains was similar.

Fig. 1. Phosphatase activities of *Citrobacter* sp. N14 and *C. freundii* NCIMB 11490 grown in phosphate-limited continuous culture. The *Citrobacter* strains were grown as described in Methods at $D = 0.5 \mu_{\max}$ (see text). Data (means $\pm$ SEM) were from several replicate determinations at the pH values shown, in buffers appropriate to each pH. For comparison the data are expressed as a percentage of the maximum activity; this corresponded to 200 units and 251 units for *Citrobacter* N14 ($\circ$) and *C. freundii* ($\bullet$), respectively.

Fig. 2. Phosphatase activities of *Citrobacter* sp. N14 and *C. freundii* NCIMB 11490 and 7271 grown in carbon-limiting continuous culture. Details as for Fig. 1. Data are means $\pm$ SEM pooled from several replicate samples from two chemostats (strains NCIMB 11490 and NCIMB 7271) or from a representative chemostat (strain N14; see text). $\bullet$, *C. freundii* NCIMB 11490; $\circ$, *C. freundii* NCIMB 7271 (solid lines, left scale); Δ , *Citrobacter* N14 (dashed line, right scale).

Acid phosphatase activities of *Citrobacter* spp. grown under carbon limitation

The *pho* regulon is generally understood to regulate transcription of genes concerned with phosphate acquisition and utilization (above) but it has been shown also that one *pho*-regulated operon (*ugp*: Wanner, 1993) is regulated by both phosphate and carbon starvation at two separate promoters (Kasahara *et al.*, 1991a). The carbon-starvation-inducible promoter is regulated by cyclic AMP and its receptor protein (CRP) which are more usually associated with carbon starvation responses (see Kasahara *et al.*, 1991a). Although the production of acid phosphatase was maximal under carbon limitation in *Klebsiella (Aerobacter) aerogenes* (Bolton & Dean, 1972), *Salmonella typhimurium* (Kier *et al.*, 1977a) and *Citrobacter* sp. N14 (Hambling *et al.*, 1987; Macaskie, 1990), in the latter case enzyme production was regulated additionally by the phosphorus status of the medium (Butler *et al.*, 1991), and cross-talk is implied here, also. A dual regulation, and additional factors such as the pH of the medium and the oxygen tension during growth (Touati *et al.*, 1987; Hallett *et al.*, 1991; Jeong, 1992), may explain the variation between batches noted above, and justify the use of continuous culture to achieve a steady-state for study. The acid phosphatase of *Citrobacter* sp. N14 is homologous to the *phoN* product of *S. typhimurium* (Macaskie *et al.*, 1994b). Regulation of *phoN* by the *phoP-phoQ* regulatory system is established (Kasahara *et al.*, 1991b); this is responsible for responses to environmental stimuli (Groisman & Saier, 1990; Miller *et al.*, 1993).

The three *Citrobacter* strains were grown in carbon-limited continuous culture (Fig. 2). In each case the acid phosphatase activity was increased as compared to the activity in glycerol-batch cultures (cf. above). It should be noted that 'acid' phosphatase activity of *K. aerogenes* and

Citrobacter sp. N14 is, more correctly, the pooled contributions of acid and neutral phosphatase activities (Bolton & Dean, 1972; Jeong, 1992). The pH-activity profiles of whole cells show activity at pH 4–8 (Fig. 2). It could be argued that the periplasmic pH differs from the pH of the bulk solution; however a similarly broad pH profile has been confirmed using purified *Citrobacter* N14 phosphatase (Jeong, 1992). An alternative argument could state that examination of an enzyme *in vitro* will not adequately portray the activity of an enzyme that may exist as a membrane-bound/periplasmic complex *in vivo*. The latter has been proposed for the PhoN phosphatase of *S. typhimurium* (Kier *et al.*, 1977b). The periplasmic localization of the enzyme is well documented in this organism (see Kier *et al.*, 1977b). During purification the *Citrobacter* sp. N14 enzyme behaved in a similar manner to that of *S. typhimurium* (Jeong, 1992). Additional confirmation of the periplasmic location of the phosphatase was provided by immunogold labelling studies (Jeong, 1992).

The phosphatase activity of strain N14 under carbon-limitation was approximately 20-fold higher than that of the two *C. freundii* strains (Fig. 2). The data for the latter were pooled from two chemostats for each; representative data from one *Citrobacter* sp. N14 chemostat are shown (specific activity was approx. 1000 units); they were indistinguishable from specific activities obtained from two other chemostats, of 911 and 1018 units, respectively. Phosphatase activity was expressed at alkaline pH under carbon limitation by strain N14 but not by the *C. freundii* strains. The flat profile of the activity of the *Citrobacter* N14 enzyme might imply that the rate-limiting step is the entry of the artificial chromophore *p*-nitrophenyl phosphate through the outer membrane. However, cellular disruption using a French pressure cell did not yield additional activity, and other studies using cells grown under carbon (glycerol) limitation in an air lift fermenter

Table 1. Effect of various inhibitors on acid, neutral and alkaline phosphatase activities of three *Citrobacter* strains

The effect of various agents at 1 mg ml⁻¹ final concentration was tested as described in Methods. The data (three determinations from each of two independent chemostats) were pooled to give a mean $\pm$ SEM of within 10–15%; mean values are shown. ND, Not determined. Numbers in bold type indicate a significant difference between the strains.

Test agent	Phosphatase specific activity (% of unsupplemented control)								
	pH 5			pH 7			pH 8.5		
	Strain: 11490	Strain: 7271	N14	Strain: 11490	Strain: 7271	N14	Strain: 11490	Strain: 7271	N14
Cd ²⁺	43.0	23.4	80.0	34.4	39.0	79.0	103.7	92.7	85.0
Zn ²⁺	26.3	18.4	81.5	14.1	9.0	2.0	81.7	66.5	111.5
Cu ²⁺	16.6	21.6	2.5	ND	ND	ND	98.9	97.5	91.0
Ni ²⁺	112.2	67.6	84.5	85.6	65.0	73.0	79.7	96.4	103.0
Co ²⁺	93.1	115.0	90.0	86.5	88.9	93.0	124.0	127.6	113.5
EDTA	96.2	101.9	97.0	103.0	94.0	106.5	100.0	117.6	97.5
Phosphate	99.3	95.9	92.0	100.3	95.7	100.5	96.8	86.5	93.0
Fluoride	18.5	21.8	9.0	33.9	39.1	32.5	70.5	73.7	25.5
Formaldehyde	83.1	79.6	71.0	73.5	76.3	69.0	84.0	106.6	72.0
Tartrate	90.5	85.7	84.0	103.6	95.6	96.5	106.1	122.0	95.0
Imidazole	107.2	97.9	109.0	98.5	98.8	93.0	82.6	87.3	86.5
Theophylline	95.9	87.6	131.0	ND	ND	ND	94.9	100.4	88.5
Phenylalanine	103.5	95.9	92.5	100.6	101.6	95.5	108.1	89.5	93.0

(Macaskie *et al.*, 1995a) or using lactose in fed-batch culture (Macaskie *et al.*, 1995b) yielded cultures with higher whole-cell phosphatase activities, of approximately 3000 and 1800 units, respectively. The use of acetate or citrate buffer to evaluate phosphatase activity at low pH is problematic since both ligands complex metals extensively. Although alkaline phosphatase activity is Mg²⁺-dependent (Ahlers, 1974; Cathala *et al.*, 1975; Linden *et al.*, 1977), the acid phosphatase of *Citrobacter* sp. N14 did not require Mg²⁺ for activity (Hambling *et al.*, 1987). As an additional check, the phosphatase activity of the three *Citrobacter* strains was confirmed to be unaffected by 1 mg EDTA ml⁻¹.

Comparison of acid-type phosphatases of *Citrobacter freundii* and *Citrobacter* sp. N14

As a confirmation that similar enzymes were being compared in the three strains, the responses of the enzymes to some diagnostic inhibitors were investigated (Table 1). Negligible inhibition by phosphate was observed; similarly tartrate, imidazole and phenylalanine had little, if any, effect. A detailed discussion of the effects of the inhibitors is outside the scope of this investigation, since the objective was to compare the overall pattern of responses to the inhibitors under test. The effect of theophylline was, however, noteworthy, since a slightly inhibitory effect was seen with both *C. freundii* strains; in contrast, the enzyme of *Citrobacter* sp. N14 was stimulated by approximately 30% at pH 5 (Table 1). A similar effect was observed previously (Hambling *et al.*, 1987).

In accordance with previous studies (Hambling *et al.*, 1987), formaldehyde and fluoride were inhibitory. At

Fig. 3. Effect of fluoride ions on whole-cell phosphatase activity. The cells were pre-incubated in the presence of NaF as described in Methods at pH 5.0 (a), pH 7.0 (b) and pH 8.5 (c). Phosphatase activity is shown as a percentage of the unsupplemented control for *C. freundii* NCIMB 11490 (●), *C. freundii* NCIMB 7271 (○) and *Citrobacter* sp. N14 (▲). The data (three determinations from each of two independent chemostats) were pooled to give a mean $\pm$ SEM of within 10–15%.

pH 5 and 7 the inhibition by formaldehyde was similar for the three strains (Table 1). The inhibitory effect of fluoride (Table 1) warranted further investigation (Fig. 3). The greater sensitivity of the *Citrobacter* sp. N14 enzyme at pH 5 was confirmed; in particular, at 5 mM F⁻ *C. freundii* retained approximately 50% of the phosphatase activity (cf. the N14 enzyme, for which > 90% of the activity was lost: Fig. 3a). At the neutral pH (Fig. 3b) the inhibition of the three strains was similar. At pH 8.5 the toxicity was less for all three strains as compared to pH 5 but strain

Fig. 4. Effect of heavy metals on whole-cell phosphatase activity. The cells were pre-incubated in the presence of heavy metals as described in Methods in the non-complexing buffers (Good *et al.*, 1966) MES (pH 5.0; a, d, g) and MOPS (pH 7.0; b, e) or the complexing buffer Tricine (pH 8.5; c, f, h). Phosphatase activity is expressed as a percentage of the unsupplemented control for cells challenged with Cd^{2+} (a, b, c), Zn^{2+} (d, e, f) or Cu^{2+} (g, h). ●, *C. freundii* NCIMB 11490; ○, *C. freundii* NCIMB 7271; ▲, *Citrobacter* sp. N14. The data (three determinations from each of two independent chemostats) were pooled to give a mean $\pm$ SEM of within 10–15%.

N14 was still the most sensitive (Fig. 3c). Lower concentrations of fluoride were not tested, although the K_i value for F^- against purified *Citrobacter* sp. N14 PhoN phosphatase isoenzymes has been determined as 1.2 mM and 1.8 mM using a Lineweaver–Burk plot (Jeong, 1992). This compares with a K_i value of 0.5 mM for the *S. typhimurium* acid phosphatase (Weppelman *et al.*, 1977).

Effects of heavy metals on phosphatase activities of *Citrobacter freundii* and *Citrobacter* sp. N14

The high Cd-tolerance of the phosphatase of the metal-accumulating *Citrobacter* sp. N14 was noted previously (Hambling *et al.*, 1987; Jeong, 1992) and was probably attributable to the low concentration of thiol-containing amino acids in the protein (Jeong, 1992). At pH 5 the enzyme of strain N14 was relatively insensitive to Cd^{2+} and Zn^{2+} as compared to the *C. freundii* strains (Fig. 4a, d),

but was more sensitive to Cu^{2+} at that pH (Fig. 4g). High tolerance to Cd^{2+} was seen also at pH 7 (Fig. 4b), but at neutral pH the *Citrobacter* N14 enzyme was more sensitive to Zn^{2+} than the enzyme of the reference strains (Fig. 4e). An apparently high tolerance to all three metals was observed for the three strains at pH 8.5 (Fig. 4c, f, h) but these results are questionable since the buffer used (Tricine) would have complexed the metals; metal complexation is necessary to suppress the formation of hydroxylated species which form at neutral and high pH (Baes & Mesmer, 1976; Hughes & Poole, 1989, 1991). The different effects of Cd^{2+} and Zn^{2+} may be attributed to the chemistry of the two metals. Cd^{2+} behaves as a 'soft' cation, with high affinity for thiol groups, while Zn^{2+} is more 'hard' in nature; it can behave also as a Lewis acid and exert a strong polarizing effect on the target molecule (Hughes & Poole, 1989). Possibly the toxic effect of this metal on the *Citrobacter* N14 phosphatase at pH 7 reflects a larger proportion of polarizable groups within the protein molecule, but why the effect should be seen at pH 7 only is not clear. This explanation may be too simplistic. Substantial hydrolysis of Cd^{2+} is largely confined to pH values above 7.0 (Hahne & Kroontje, 1973; Baes & Mesmer, 1976), while the hydrolysis of Zn^{2+} and Cu^{2+} begins at lower pH values. The pK_a values of the aqua cations of Cu(II) and Zn(II) are 8.0 and 9.0, respectively (Hughes & Poole, 1991) and the formation of hydroxylated species accordingly begins at a lower pH with Cu^{2+} than with Zn^{2+} (Baes & Mesmer, 1976). It is possible that an aqua carbonato species (Hughes & Poole, 1991) may actually constitute the toxic agent at neutral pH in the case of Zn^{2+} , and at pH 5 in the case of Cu^{2+} (Fig. 4e, g). It was not possible to assess the toxicity of Cu^{2+} at pH 7 in the non-complexing (Good *et al.*, 1966) buffer MOPS, since $\text{Cu}(\text{OH})_2$ precipitated out of the solution, but the similarity of the pattern of the Cu^{2+} toxicity at pH 5 to that of Zn^{2+} toxicity at pH 7 for the three strains should be noted. In each case the *Citrobacter* sp. N14 enzyme was the most susceptible. This is in contrast to the situation where the metals would have been present as the free divalent cations (Cd^{2+} at pH 5 and 7, and Zn^{2+} at pH 5) or as the $\text{Cd}(\text{OH})^+$ or $\text{Zn}(\text{OH})^+$ species (Baes & Mesmer, 1976); the pK_a value of $\text{Cd}(\text{II})$ is 9.9 (cf. above) and accordingly the Cd^{2+} and $\text{Cd}(\text{OH})^+$ species persist at pH 7 (Baes & Mesmer, 1976). Further interpretation is not possible with the information available, but the pattern of metal toxicity may reflect differences in the structure, composition, charge or degree of hydration of the enzymes or, indeed, their possible localization in association with membranes or other high molecular mass structures *in vivo* (Kier *et al.*, 1977b; B. C. Jeong and others, unpublished).

Phosphatase activity and Cd-sensitivity of *C. freundii* 'trained' to tolerate Cd^{2+}

Using serial subcultures in progressively increasing concentrations of Cd^{2+} (see Methods) *C. freundii* NCIMB 11490 was 'trained' to Cd resistance. The growth rate after several subcultures at test concentrations of 50 and 100 $\mu\text{g Cd}^{2+} \text{ ml}^{-1}$ was half that of the control value using

Fig. 5. Phosphatase activity of *C. freundii* NCIMB 11490 'untrained' (♦) and 'trained' to Cd resistance at 50 µg ml⁻¹ (●) and 100 µg ml⁻¹ (○). The cells were cultured in chemostats supplemented with Cd²⁺ as appropriate at $D = 0.5 \mu_{\max}$ as determined under appropriate conditions (see text). For growth in the Cd-'trained' cultures the $\mu_{\max}$ corresponded to $D = 0.28 \text{ h}^{-1}$ at both concentrations, compared to 0.55 h^{-1} in the 'untrained' control. Data are from cells from two chemostats (see text).

Fig. 6. Cd sensitivity of phosphatase activity of *C. freundii* NCIMB 11490 'trained' to Cd resistance (100 µg Cd²⁺ ml⁻¹) and challenged with Cd²⁺ at (a) pH 5 (MES buffer) and (b) pH 7 (MOPS buffer). ●, Untrained *C. freundii*; ○, *C. freundii* trained to resistance to 100 µg Cd²⁺ ml⁻¹.

Cd-unstressed cells. This was assigned as a new value of $\mu_{\max}$. Continuous cultures of 'trained' *C. freundii* NCIMB 11490 were maintained at $D = 0.5 \mu_{\max}$. The phosphatase specific activity of the 'trained' cultures was apparently reduced as compared to the untrained culture (maintained at $D = 0.5 \mu_{\max}$ as determined in 'untrained' cultures: Fig. 5), in contrast to the effect predicted if phosphatase overproduction was selected as a mechanism of metal detoxification (see Introduction). The pH profile remained constant. It was possible that selection within the chemostats for a metal-tolerant strain effectively increased the $\mu_{\max}$ value during the experiment, such that $D (= \mu)$ was not a constant proportion of $\mu_{\max}$. This could mean that the relative growth rate would be effectively decreasing with time at a constant D with respect to $\mu_{\max}$ (i.e. a non-steady-state system). The growth-rate-dependence of phosphatase production has been noted previously (Hambling *et al.*, 1987). Although this was not investigated, inspection of Fig. 5 shows this to be unlikely. The phosphatase activity of the cultures 'trained' to tolerate 100 µg Cd²⁺ ml⁻¹ was less than that of cultures 'trained' to 50 µg ml⁻¹; the latter culture might have been expected to select for metal resistance more rapidly (i.e. to have been maintained at a progressively lower growth rate relative to $\mu_{\max}$), since the concentration of Cd²⁺ was less. For the above argument to apply, the culture 'trained' to 50 µg Cd²⁺ ml⁻¹ should have had less phosphatase activity than the culture 'trained' to 100 µg ml⁻¹, yet the converse was observed (Fig. 5). It was concluded that, in contrast to a role for the phosphatase in metal resistance (see Introduction), continued exposure to Cd²⁺ reduces phosphatase production in *C. freundii*. The metal toxicity to the enzyme following 'training' was also investigated in order to discount the

possibility of production of an altered enzyme which was sensitive to Cd-toxicity.

Sensitivity of *C. freundii* phosphatase to Cd²⁺ before and after 'training' of the cells to Cd tolerance

No evidence was obtained for altered Cd resistance of the phosphatase enzyme during 'training' of *C. freundii* to Cd²⁺ (Fig. 6). The Cd-sensitivity of the phosphatase of the 'untrained' *C. freundii* was largely pH-independent, discounting pH-dependent Cd speciation effects (Fig. 6a, b). Development of Cd-tolerance by the Cd-'trained' *C. freundii* gave altered responses of cellular phosphatase activity. At solution pH values of 5 and 7 the Cd resistance of the enzyme was increased (Fig. 6a) and decreased (Fig. 6b) respectively. It is not easy to interpret the biological significance of this, since the pH of the periplasm in juxtaposition to the enzyme is not known and would be difficult to determine. The apparently increased sensitivity of the enzyme to Cd at neutral pH was not significant at 100 µg Cd²⁺ ml⁻¹ (Fig. 6b) and could not have accounted for the effect seen in Fig. 5.

Acid phosphatase activity in *Citrobacter* spp. and other *Enterobacteriaceae*

The evidence presented here does not suggest that acid phosphatase overproduction is a metal detoxification response to deposit heavy metals harmlessly at the cell

surface. Attempts to enhance phosphatase production by *C. freundii* by 'training' the cells to tolerance to Cd^{2+} were unsuccessful. Phosphatase overproduction by *Citrobacter* N14 does, however, appear to be atypical of the genus; indeed, the *phoN* gene encoding this enzyme (Macaskie *et al.*, 1994b) is absent from *C. freundii* and also from *E. coli* (Kasahara *et al.*, 1991b; Groisman *et al.*, 1992), although *E. coli* has the PhoP–PhoQ regulatory system (Kasahara *et al.*, 1992). *phoN* may be transferred laterally in the environment (Groisman *et al.*, 1992), although no plasmids were found in strain N14. The question arises as to the identity of the acid phosphatase expressed in *C. freundii* strains under carbon limitation (which promotes expression of *phoN*; Kier *et al.*, 1977a). Acid phosphatase activity is clearly seen in *C. freundii* (this communication) and *E. coli* (below), yet *phoN* is apparently absent (see above). This discrepancy was also noted by Rossolini *et al.* (1994), who demonstrated acid phosphatase activity in *E. coli* grown on carbon sources other than glucose. Recent work (Thaller *et al.*, 1994, 1995a, b) suggests that enteric bacteria can produce two distinct periplasmic acid phosphatases, classified as type A (typified by PhoN of *S. typhimurium* and *Citrobacter* sp. N14, and PhoC of *Morganella*) or class B (e.g. the NapA enzyme of *Morganella morganii*; Thaller *et al.*, 1995a). The latter type, apparently widespread amongst enteric bacteria, including *Citrobacter* spp. (Thaller *et al.*, 1995b), characteristically requires Mg^{2+} for activity and is inhibited by EDTA. The latter was not observed for the carbon-starved *C. freundii* enzyme (Table 1). In addition, although the NapA of *M. morganii* is stimulated by Co^{2+} and Zn^{2+} (Thaller *et al.*, 1995a) this was not observed here for *C. freundii*. Conversely, the *M. morganii* class B acid phosphatase is fluoride resistant (Thaller *et al.*, 1995a), but the acid phosphatase of both strains of *C. freundii* was fluoride sensitive (Fig. 3), a property more in accordance with PhoN. Clearly the enterobacterial phosphatases warrant more extensive examination. In this context it should be noted that although acid phosphatase was well documented in *Klebsiella* (Bolton & Dean, 1972), and subsequently identified in several strains as type A (M. C. Thaller, personal communication) *phoN* is thought to be absent from this organism (e.g. Groisman *et al.*, 1989). Further clarification is clearly required.

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