

Patterns of Phosphatase Activity among Enterobacterial Species

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The phosphatase activities of cells of representative enterobacterial species were studied after cells were grown in a chemically defined medium with variations in the amounts of phosphates and in pH (4.00 to 9.00). All strains of a given species demonstrated similar acid phosphatase activities after growth in the presence or absence of phosphates. In contrast, alkaline phosphatase was produced only by some species, and it was also strongly influenced by the presence of either glycerophosphate or P_i in the medium. There were four patterns of phosphatase activity, depending on the properties of the alkaline phosphatase. Phosphatase activity can be a valuable characteristic for inclusion in tests for the identification of enterobacteria.

Phosphatase activity among enterobacteria has been the subject of considerable interest during past decades (1–4), but most work dealt with only a few strains of different species and used different assay conditions, producing results that were often contrasting and not easy to compare (6, 9, 10, 18, 22, 25–27). In fact, among enterobacteria only *Escherichia coli* and *Aerobacter* (*Klebsiella*) *aerogenes* have been analyzed for production of acid and alkaline phosphatases in the same strains (3, 23). One or the other of these enzymes was examined in detail in a few species and ignored in others (2, 5, 12, 13, 18, 25, 27). Consequently, our knowledge of enterobacterial phosphatases is incomplete. Moreover, the information that we have regarding phosphatases derives from very few strains of a species.

Recently, phosphatase activity was shown to be a constant property of colonies of all the major species of the family *Enterobacteriaceae* (17). A novel method, called methyl-green phosphatase (MGP) (15), used different substrates in a plate medium to evaluate the phosphatases of colonies of different species. The technique allowed the separation of the *Proteus-Providencia* group from other enterobacteria and the identification of *Providencia stuartii* strains (15, 16). Therefore, it is possible that the specific properties of the phosphatases and the levels of activity may be characteristic of various species of the group and may contribute to species identification, as has already been done for other bacteria (7, 11, 19) but which has been done for only a few enterobacterial species (14, 16).

We have studied the phosphatase activities of the enterobacterial species most commonly isolated from clinical specimens by growing them in a chemically defined medium either containing or not containing different amounts of phosphates in buffers at pHs ranging from 4.00 to 9.00.

MATERIALS AND METHODS

Strains. A total of 60 strains, belonging to 16 enterobacterial species commonly isolated from clinical specimens, were tested for phosphatase activity at different pHs and with different amounts of P_i in the growth medium, which was or was not supplemented with glycerophosphate (G3P). Enterobacterial species were isolated from clinical speci-

mens in our laboratory and were identified by conventional methods (8). Species of the tribe *Proteeae* were identified as reported before (14). *Escherichia coli* ATCC 25922 was also used.

Chemically defined medium for phosphatase assay. The composition of the chemically defined medium (CDM), deprived of P_i and used for growing enterobacteria and assaying phosphatase activity, was as follows (in milligrams per liter): glucose, 1,000; NaCl, 5,000; $MgSO_4 \cdot 7H_2O$, 200; $CaCl_2$, 200; KCl, 400; L-methionine, 15; L-glutamine, 292; L-lysine, 72.5; L-valine, 46; L-histidine, 42; tryptophan, 10; L-leucine, 52; L-arginine, 126; L-cystine, 24; L-phenylalanine, 32.50; L-threonine, 48; L-tyrosine, 36; L-isoleucine, 52; biotin, 1; calcium pantothenate, 1; folic acid, 1; nicotinamide, 1; thiamine, 1; inositol, 2; pyridoxal, 1; and riboflavin, 0.1. All of the substances were obtained from BDH Italia (Milan, Italy) and were dissolved in distilled water and sterilized by filtration. The pH was adjusted to 7.4. G3P (Carlo Erba, Milan, Italy) was added to the medium at a concentration of 30 mg/liter when needed in order to enhance the rate of bacterial growth.

Determination of phosphatase activity. Acid phosphatase (ACP) and alkaline phosphatase (ALP) activities were detected by a modification of the method described by Torriani (23). Bacteria were grown for 18 h in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) at 37°C. The cells were then harvested by centrifugation at $3,150 \times g$ for 20 min and washed twice with saline. Bacteria were seeded in CDM containing different amounts of P_i (from 0 to 0.5 M, in a mixture of monobasic and dibasic phosphate); the pH was always adjusted to 7.4. After an additional 24 h of growth at 37°C, the cells were centrifuged, washed twice with saline, and resuspended at a final optical density of 0.200 at 620 nm. Toluene (0.2 ml per ml of cell suspension) was added, left for 15 min after a gentle shaking, and then removed; pHs of 1-ml fractions of the cell suspension were regulated to between 4.0 and 9.0 by adding either acetate buffer or Tris buffer. Five hundred micrograms of *p*-nitrophenyl phosphate was added to each tube, and the suspensions were incubated for 3 to 15 h at 37°C (except in the case of *Providencia stuartii*, which was incubated for 1 h). At the end of the incubation time, 0.2 ml of 2 N NaOH was added to each tube to stop the reaction. The cells were pelleted by centrifugation, and the supernatant was analyzed for *p*-nitrophenyl release in a spectrophotometer at 420 nm.

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TABLE 1. Effect of G3P on the expression of phosphatase activity by different enterobacterial species

Species	Phosphatase activity ^a			
	With G3P at pH:		Without G3P at pH:	
	5.0	9.0	5.0	9.0
<i>Escherichia coli</i>	+	+	+	+
<i>Serratia marcescens</i>	+	+	+	+
<i>Proteus vulgaris</i>	+	+	+	+
<i>Klebsiella pneumoniae</i>	++	—	++	—
<i>Klebsiella oxytoca</i>	++	—	++	—
<i>Citrobacter freundii</i>	+	—	++	—
<i>Salmonella typhi</i>	+	—	+	—
<i>Salmonella</i> serovar wien	+	—	+	—
<i>Morganella morganii</i>	+++	—	+++	—
<i>Proteus mirabilis</i>	+	—	+	—
<i>Providencia stuartii</i>	+++	—	+++	—
<i>Providencia alcalifaciens</i>	+	+	+	—
<i>Serratia liquefaciens</i>	+	+	+	—
<i>Yersinia enterocolitica</i>	+	—	+	+
<i>Providencia rettgeri</i>	+	—	+	+
<i>Enterobacter cloacae</i>	+	—	+	+

^a Activity was determined in CDM lacking P_i. +, ++, and +++ indicate increasing amounts of phosphatase activity produced; — indicates the absence of phosphatase activity.

One unit of phosphatase activity was the amount of enzyme able to liberate 1 μ mol of *p*-nitrophenyl/h/ml of the bacterial suspension. Standard curves were obtained by using either commercial potato ACP or ALP from bovine intestinal mucosa (SIGMA Chimica, Milan, Italy) at various incubation times.

RESULTS

In order to study the effect of phosphates on phosphatase production, each species considered in this study was grown in CDM devoid of P_i in the presence or the absence of G3P

at two different pHs, namely, 5.0 and 9.0. Table 1 shows the effect of G3P on the phosphatase activities of the various species studied. ALP was expressed by only a few species. On the basis of ALP production and the effect of G3P on it, four main patterns of phosphatase activity could be observed among the enterobacterial species tested. *Escherichia coli*, *Serratia marcescens*, and *Proteus vulgaris* expressed detectable amounts of ALP in both the presence and absence of G3P, and ALP was not affected by the presence of G3P in the medium. *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Salmonella typhi*, *Salmonella* serovar wien, *Morganella morganii*, *Proteus mirabilis*, *Providencia stuartii*, and *Citrobacter freundii* always lacked ALP, but produced various amounts of ACP. *Yersinia enterocolitica*, *Enterobacter cloacae*, and *Providencia rettgeri* had ALP activities in the absence of G3P which were totally inhibited by addition of G3P. *Serratia liquefaciens* and *Providencia alcalifaciens* appeared to be endowed with ALP only in the presence of G3P.

A number of strains of each species were studied for phosphatase production at pHs from 4.00 to 9.00 and in the presence of P_i concentrations from 0 to 0.5 M. It is evident that within a species, both ACP and ALP generally present quite homogeneous characteristics in terms of properties, amount of enzyme produced, optimal pH, and requirement of P_i for optimal enzyme production (Table 2). In contrast, the strains of the various species differed from each other in one or several of those parameters. *Escherichia coli* generally expressed a marked ALP activity ranging from 6 to 20 mU/ml/h which peaked at pH 9.0, as did *Proteus vulgaris*, while *S. marcescens* had an ALP activity that peaked at pH 8.5 and a weak ACP activity (from 5 to 10 mU) that peaked at pH 5.5. The highest production of phosphatase was reached for these three species in the presence of P_i concentrations of 0.1 M. *Serratia marcescens* produced moderate amounts of ALP (from 8 to 41 mU) which were only partially repressed by P_i. ACP of *Serratia marcescens* ranged from 9 to 16 mU and peaked at 4.5. *Proteus vulgaris* produced a larger amount of ACP (from 23 to 86 mU) than of ALP (8 to 10 mU). ALP was completely repressed by P_i. *Proteus*

TABLE 2. Main properties of phosphatase activities of different enterobacterial strains

Species (no. of strains)	Phosphatase activity ^a at:		Optimal pH		Optimal P _i concn (M) for phosphatase activity at:	
	Alkaline pH (range)	Acid pH (range)	Alkaline	Acid	Alkaline pH	Acid pH
<i>Escherichia coli</i> (7)	11.5 (6–20)	9.7 (5–10)	9.0	5.5	0	0.1
<i>Serratia marcescens</i> (3)	23.0 (8–41)	12.3 (9–16)	8.5	4.5	0	0.1
<i>Proteus vulgaris</i> (3)	9.0 (8–10)	44.3 (23–86)	9.0	7.5 ^b	0	0.1
<i>Providencia alcalifaciens</i> (4)	34.2 (24–50)	93.0 (82–99)	9.0	4.5	0	0.1
<i>Serratia liquefaciens</i> (3)	32.0 (25–41)	9.0 (7–11)	9.0	5.5	0	0.02
<i>Providencia rettgeri</i> (3)	17.6 (11–23)	61.3 (47–90)	9.0	4.5	0	0.1
<i>Enterobacter cloacae</i> (3)	12.0 (8–17)	5.0 (3–9)	9.0	4.5	0	0.1
<i>Yersinia enterocolitica</i> (3)	4.6 (3–7)	12.0 (8–19)	9.0	<4.0	0	0
<i>Klebsiella pneumoniae</i> (4)		974.2 (797–1,120)		5.5		0.1
<i>Klebsiella oxytoca</i> (3)		876.0 (698–910)		5.5		0.1
<i>Salmonella typhi</i> (4)		54.7 (40–62)		4.5		0.5
<i>Salmonella</i> serovar wien (3)		32.3 (18–51)		4.5		0.5
<i>Morganella morganii</i> (3)		23,406.6 (10,200–40,520)		5.5		0.5
<i>Proteus mirabilis</i> (3)		69.0 (25–100)		6.5		0.02
<i>Providencia stuartii</i> (4)		87,932.5 (78,000–101,200)		5.5		0.1
<i>Citrobacter freundii</i> (3)		4.6 (1–8)		4.5		0.1

^a Phosphatase activity is expressed in milliunits per milliliter per hour. The strains were grown in a medium supplemented with G3P.

^b This phosphatase would be better considered a neutral phosphatase.

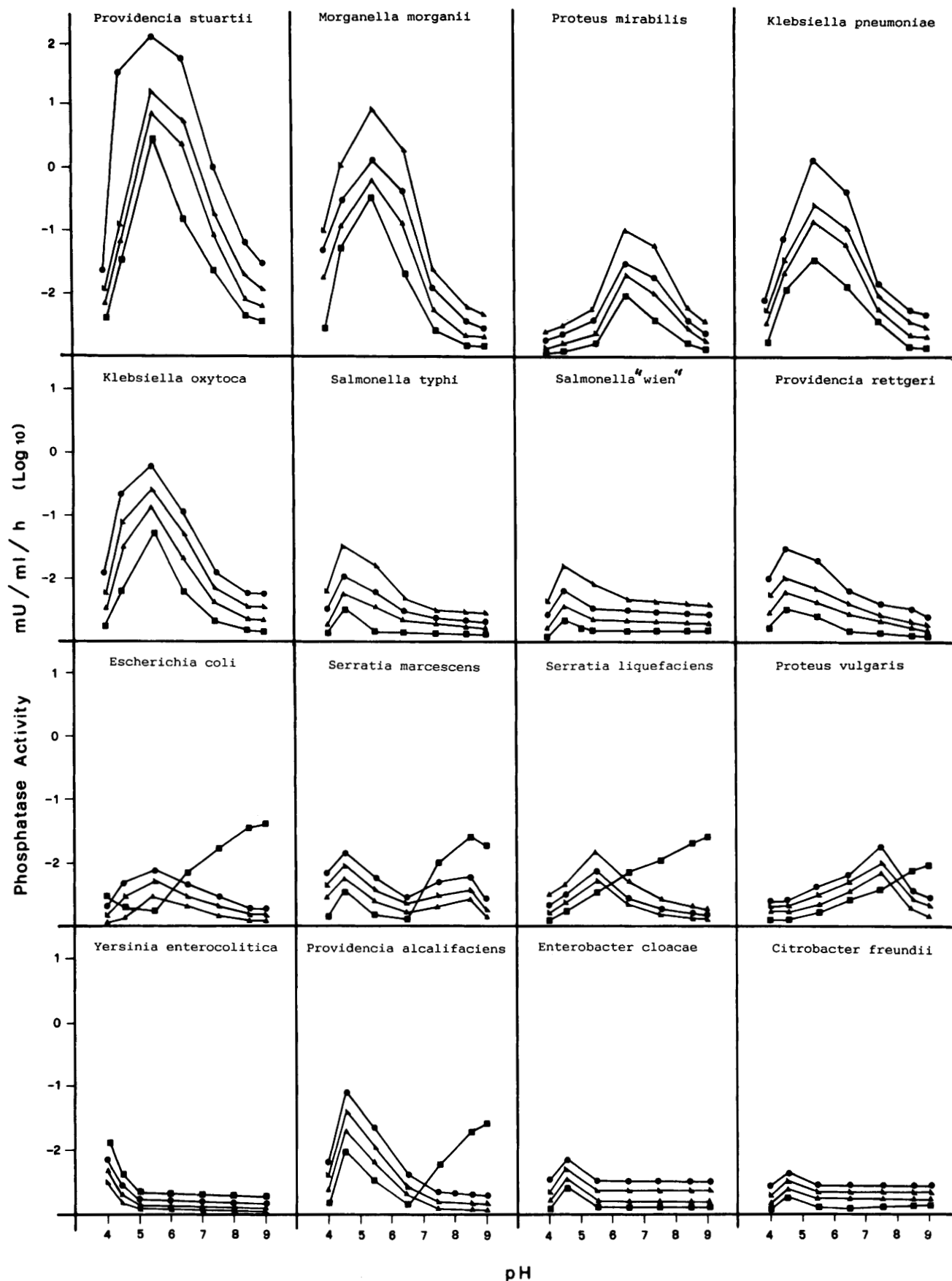


FIG. 1. Phosphatase activity profiles of enterobacterial type strains grown on a chemically defined medium supplemented with G3P under conditions of different pHs and P_i concentrations. ■, absence of P_i ; ▲, 0.02 M P_i ; ●, 0.1 M P_i ; ◆, 0.5 M P_i .

vulgaris did not express a real ACP, but presented a peak of phosphatase activity at pH 7.5, which showed a behavior similar to that of ACP from all of the other species. *Providencia alcalifaciens* produced moderately elevated amounts of both ALP and ACP (24 to 50 and 82 to 99 mU, respectively). ALP activity peaked at pH 9.0, as did the ALP activity of *Serratia liquefaciens*. ACP activity peaked at pH 4.5 and was induced by P_i up to a concentration of 0.1 M. The ALP of *Serratia liquefaciens* was produced in amounts from 7 to 11 mU. ACP activity ranged from 25 to 41 mU, peaked at pH 5.5, and was stimulated by small amounts of P_i (0.02 M). The ALP of *S. liquefaciens* and *P. alcalifaciens* was completely repressed by small amounts of P_i . *Enterobacter cloacae*, *Yersinia enterocolitica*, and *Providencia rettgeri* produced a P_i -repressible ALP only in medium deprived of G3P. The ACP of *Enterobacter cloacae* had a weak activity (3 to 9 mU) that peaked at pH 4.5, as did that of *Providencia rettgeri*, and was induced by 0.1 M P_i , similar to the ACP of *Providencia rettgeri*. *Yersinia enterocolitica* behaved peculiarly; it produced a P_i -inhibitable ALP with an activity of 3 to 7 mU and an ACP with an activity of 8 to 19 mU which peaked at a pH lower than 4.0 and showed its maximum activity at a P_i concentration of 0 M. *Providencia rettgeri* has a strong ACP stimulated by P_i up to a concentration of 0.1 M; ALP was produced in amounts from 11 to 23 mU, and ACP was produced in amounts from 47 to 90 mU. *Klebsiella*, *Morganella* and *Providencia stuartii* strains were always devoid of ALP in both the presence and the absence of G3P but were very strong ACP producers. Productivity ranged from 698 to 1,120 mU/ml/h for the *Klebsiella* species, while it was 10,200 to 40,520 mU/ml/h in *Morganella morganii* and 78,000 to 101,200 mU/ml/h in *Providencia stuartii*. The ACP of *Morganella morganii* peaked at pH 5.5 and was progressively induced by P_i to reach a maximum at 0.5 M P_i . *Providencia stuartii* was by far the best ACP producer. It peaked at pH 5.5 and was induced by a P_i concentration of 0.1 M. *Salmonella* serovar wien and *Proteus mirabilis* produced less ACP than *P. stuartii*, *M. morganii*, and the *Klebsiella* species (ranges, 18 to 62 mU and 25 to 100 mU, respectively). *Salmonella* ACP peaked at 4.5 in most strains and was induced by increasing P_i concentrations up to 0.5 M. ACP of *Proteus mirabilis* peaked at pH 6.5 and was stimulated by small amounts of P_i . *Citrobacter freundii* was a very poor phosphatase producer (1 to 8 mU/ml/h). Its enzyme peaked at pH 4.5 and was stimulated by P_i . The phosphatase properties of each enterobacterial species tested are summarized in Fig. 1.

DISCUSSION

The present work shows that different strains of the same enterobacterial species share the same pattern of phosphatase activity and that different species exhibit different patterns of activity. Furthermore, several peculiar properties of some strains have been clarified; in fact, ALP has been shown to be produced by many but not all enterobacterial species and is generally repressed by P_i , except in the species *Serratia marcescens*. In addition, some species show ALP activity only in the presence of G3P, while others have ALP activity that is repressed by G3P.

Acid phosphatases were detected in all of the species examined. In all cases they were not affected by P_i and were only slightly influenced by G3P. The differences observed in the phosphatase activities of the different species were both qualitative and quantitative.

By considering the type of phosphatase, the amount of

enzyme produced, the peak pH, the optimal P_i concentration for phosphatase activity, the effect of P_i on phosphatase expression, and the action of G3P on ALP production, all species could be distinguished from each other, with the exception of *Salmonella typhi*, which appeared to be very similar to *Salmonella* serovar wien, and *Klebsiella pneumoniae*, which closely resembled *Klebsiella oxytoca*. All other species had at least one major qualitative characteristic which enabled them to be differentiated from the other species. This fact is very useful, and because of it phosphatase activity is an important property to be considered for developing novel methods for the definition and identification of some enterobacterial species.

The finding that phosphatase activity is distinctive in the various enterobacterial species (with very few exceptions) could give further taxonomic and practical value to the methyl-green phosphatase test (15) for differentiation of some enterobacterial species in microbiology laboratories. A method has already been developed (14) for the identification of *Providencia stuartii* and *Morganella morganii* on the basis of analysis of phosphatase activity, and a selectively differentiating medium for the rapid detection of these species on agar plates has been proposed (20). In addition, the phosphatase of *Providencia stuartii* has already been cloned, and a probe that promptly identifies this species has been prepared (21).

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