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Repression of Penicillin G Acylase of *Proteus rettgeri* by Tricarboxylic Acid Cycle Intermediates

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The regulation of the penicillin acylase in *Proteus rettgeri* ATCC 31052 was compared with that of the enzyme in *Escherichia coli* ATCC 9637. Unlike the *E. coli* acylase, the *P. rettgeri* enzyme was not induced by phenylacetic acid, nor was it subject to catabolite repression by glucose. The *P. rettgeri* acylase appears to be expressed constitutively but is subject to repression by the C_4-dicarboxylic acids of the tricarboxylic acid cycle, succinate, fumarate, and malate.

Penicillin acylase (EC 3.5.1.11) catalyzes the hydrolysis of penicillin G (Pen G), forming 6-aminopenicillanic acid and phenylacetic acid (PAA) as shown in Fig. 1. This enzyme is of commercial importance since one of its products, 6-aminopenicillanic acid, is a key intermediate for the synthesis of numerous semisynthetic penicillins. Penicillin acylases are found in both eucaryotic and procaryotic microorganisms (9). Two of the many procaryotes which produce penicillin acylases are *Escherichia coli* W ATCC 9637 and *Proteus rettgeri* ATCC 31052. A number of physiological studies on the regulation of this enzyme have been carried out with the *E. coli* strain (17). The *E. coli* penicillin acylase is induced by PAA, and it is also catabolite repressed by glucose (6, 13). In addition, the *E. coli* enzyme is expressed when the cells are grown at suboptimal temperatures (28°C or below) (17). No expression of enzyme activity occurs when the cells are grown at 37°C. In contrast to the *E. coli* strain, very little was known about the regulation of the acylase from *P. rettgeri*. It was not known whether the *P. rettgeri* enzyme was inducible, affected by catabolite repression, or affected by ammonia repression.

This communication presents evidence that the regulation of the *P. rettgeri* penicillin acylase is quite different from the *E. coli* system. The *P. rettgeri* enzyme was not induced by PAA, and it was not subject to catabolite repression by glucose. The enzyme appears to be expressed constitutively but repressed by the C_4-dicarboxylic acids of the tricarboxylic acid cycle, succinate, fumarate, and malate.

MATERIALS AND METHODS

Microbiological procedures. *E. coli* W ATCC 9637 and *P. rettgeri* ATCC 31052 were used throughout this study. Stanier's standard mineral base medium (15) supplemented with 0.2% of an appropriate carbon source was used to propagate either strain. To monitor the formation of acylase during growth of *P. rettgeri*, the cells were grown at 28°C in 2-liter Erlenmeyer flasks containing 800 ml of medium. Aeration was accomplished by shaking the flasks in a gyratory shaker. Samples of whole broth were withdrawn at various times during the growth period for analysis. When the pH was maintained constant throughout the growth period, the cells were grown at 28°C in a 7-liter Microferm fermentor (New Brunswick Scientific Co.) equipped with a pH probe and titrator (Rainin Co.).

Analytical procedures. Penicillin acylase activity was assayed either by high-pressure liquid chromatography after the hydrolysis of Pen G or colorimetrically after the hydrolysis of *N*-(3-carboxy-4-nitrophenyl)-phenylacetamide (NCNPA) (11).

(i) Hydrolysis of Pen G. Reversed-phase high-pressure liquid chromatography was carried out on a Waters Associates μ-C18 column (250 by 4 mm [inner diameter]). The mobile phase was made up of 50 volumes of 50 mM acetic acid and 50 volumes of methanol. With a flow rate of 1.2 ml/min, the retention times for Pen G and the hydrolytic products, PAA and 6-aminopenicillanic acid, were 11.7, 7.5, and 2.7 min, respectively. The concentration of PAA formed from the enzymatic hydrolysis of Pen G was determined at 254 nm by measuring relative peak heights obtained from the high-pressure liquid chromatographic chart recorder with standard solutions of PAA.

(ii) Hydrolysis of NCNPA. Hydrolysis of NCNPA resulted in the formation of 2-nitro-5-aminobenzoic acid. A Bausch & Lomb Spectronic 20 spectrophotometer was used to measure the rate of product formed in filtrates derived from whole-cell incubations. The concentration of 2-nitro-5-aminobenzoic acid in the filtrate was calculated from a standard curve which plotted the concentration of standard solutions of 2-nitro-5-aminobenzoic acid against absorbance at 405 nm. In crude cellular extracts the formation of 2-nitro-5-aminobenzoic acid was measured kinetically, using a Varian Superscan 3 spectrophotometer. A molar extinction coefficient of 9,090 liters mol^{-1} cm^{-1} was used to quantitate the amount of 2-nitro-5-aminobenzoic acid formed (11).

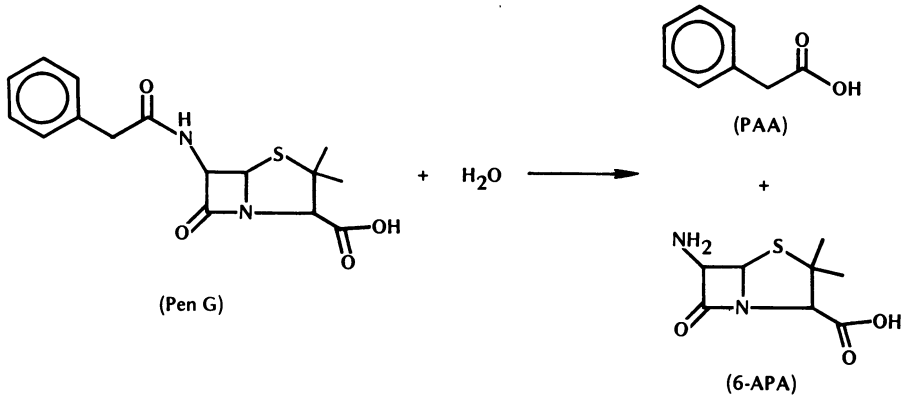

FIG. 1. Formation of PAA and 6-aminopenicillanic acid (6-APA) by penicillin acylase.

(iii) Whole-cell assays. Washed whole cells were incubated at 37°C in a 50 mM potassium phosphate solution at pH 7.5 containing either 15 mM Pen G or 3 mM NCNPA. After the incubation period the cells were removed by centrifugation, and the broth was filtered through a 0.45-μm membrane filter (Millipore Corp.). The product was quantitated by high-pressure liquid chromatography or spectrophotometrically as described above. Enzyme activity in whole cells is expressed in micromoles of product formed per hour per milligram of dry weight. Dry weights were calculated turbidimetrically from a standard curve which plotted dry weights against turbidity.

(iv) Cell-free assays. Crude cellular extracts were prepared from washed cells by suspending them in a small volume of 50 mM potassium phosphate buffer at pH 7.5 and subjecting the thick slurry to sonic disruption (Branson model J-17A Sonifier). The temperature was kept below 10°C during the treatment. Unbroken cells and cell debris were removed by centrifugation for 30 min (30,000 × *g*), and the clear supernatant was assayed for acylase activity. Acylase activity was measured as described above, using NCNPA as the substrate. Specific activities of cell extracts are expressed in micromoles of product formed per hour per milligram of protein. Protein was determined by the spectrophotometric method of Groves et al. (8), using bovine serum albumin as the protein standard.

(v) Determination of glucose. Glucose was determined on culture fluid filtrates, using a Beckman glucose analyzer according to the procedure described in the operating manual.

(vi) Synthesis of NCNPA. NCNPA was synthesized by a modification of the procedure of Kutzbach and Rauenbusch (11). Technical-grade (70%) 2-nitro-5-amino-benzoic acid (39 g; 150 mmol) was dissolved in 200 ml of 50:50 acetone-water at 15°C, and the solution was adjusted to pH 7 with 6 N NaOH. A solution of phenylacetyl chloride (23.3 g; 150 mmol) in 20 ml of acetone was added dropwise with concurrent addition of 6 N NaOH to maintain the pH at 7. After the addition was complete and the pH had stabilized, an equal volume of ethyl acetate was added, and the pH was lowered to 1.7 with 6 N HCl. The aqueous layer was discarded, and the ethyl acetate was removed under reduced pressure. The residue was dissolved in 300 ml of methanol and treated with 100 ml of Dowex 50W-X8. The resin was filtered off, and the methanol evaporated. The product was recrystallized and carbon treated three times in ethanol-water to yield 9 g (20%) of NCNPA, mp 206 to 207°C (literature, 210°C). Analysis based on the empirical formula $C_{15}H_{12}N_2O_5$ (300.27 g) gave the following results: percent calculated, C 60.00, H 4.03, N 9.33; percent found, C 59.60, H 3.93, N 9.26.

RESULTS

To obtain preliminary information on the regulation of the *P. rettgeri* penicillin acylase, whole-cell enzyme assays were carried out on cells grown under different conditions, and the results were compared with the *E. coli* strain (Table 1). When *E. coli* cells were grown in succinate at 37°C, no acylase activity was detected even in the presence of the inducer. The *P. rettgeri* acylase-producing strain did not grow at 37°C. The *E. coli* cells expressed acylase activity when grown in succinate at 28°C as long as the inducer was incorporated in the growth medium. Under these same growth conditions the *P. rettgeri* strain expressed basal levels of enzymatic activity. Phenylmalonate, like PAA, did not serve as inducer for the *P. rettgeri* strain. However, when glucose was incorporated in the growth medium, a severalfold increase in enzyme specific activity was observed even though this strain did not grow in glucose as its only source of carbon and energy.

To investigate further this enhancement of acylase activity by glucose, *P. rettgeri* cellular extracts were prepared from cells which had been grown in the presence and absence of glucose. Extracts prepared from cells grown in the glucose-supplemented medium contained higher specific activity than extracts prepared from cells grown in succinate alone (Table 1).

No evidence of enzyme activation was observed when extracts from succinate-grown

TABLE 1. Comparison of penicillin acylase expression in *E. coli* and *P. rettgeri*

Supplement added to growth medium[a]	Growth temp (°C)	Sp act (μmol of product formed/h per mg, dry wt)[b]	
		E. coli	*P. rettgeri*
None	37	0.0	No growth
Phenylacetate, 0.03%	37	0.0	No growth
Phenylacetate, 0.03%	28	1.1	0.2
None	28	0.0	0.6
Phenylmalonate, 0.03%	28		0.3
Glucose, 0.2%	28		2.5
None	28		0.5[c]
Glucose, 0.2%	28		2.1[c]

[a] The cells were grown for 18 to 20 h in Stanier's standard mineral base medium containing 0.2% succinate as carbon source and supplemented as indicated.

[b] Specific activity was determined in whole cells as described in the text, using Pen G as substrate.

[c] Specific activity was determined in cell extracts as described in the text, using NCNPA as substrate. Expressed as given except per milligram of protein.

cells were assayed in the presence of glucose or after incubation of these extracts for several hours with the sugar. In addition, no inhibition of enzyme activity was observed when the extract containing high-specific-activity acylase was assayed in the presence of succinate (10%). Furthermore, incubation of the high-activity extract with succinate or with the low-activity extracts for over 1 h had no effect on the specific activity of the acylase. Overnight dialysis of the extracts had no effect on enzyme activity.

The formation of the enzyme was monitored during growth. Figure 2 presents the results obtained when the cells were grown in succinate alone (Fig. 2A) and when the cells were supplemented with glucose (Fig. 2B). When cells were grown in succinate as the only source of carbon, the specific activity of the enzyme stayed low during the entire growth phase, and a steady increase in pH occurred (from 6.9 to 8.2). When the succinate growth medium was supplemented with glucose, a completely different pattern was observed. The enzyme activity remained low and constant during the exponential growth phase but increased dramatically once the cells reached the stationary phase. The glucose level in the culture fluid decreased at a constant rate during the growth of the organism. The pH rose but to a lesser extent (from 6.9 to 7.5), apparently as the result of some metabolic acid released during the fermentation of the sugar.

To determine whether the enhancement of activity by glucose was the result of the sugar controlling the pH of the growth medium, cells were grown in succinate, and the pH was held constant at 7. Under these conditions the enzyme level remained low during the entire growth period, even after additional succinate was made available to the cells upon entering the stationary phase of growth.

To investigate the stimulation of acylase production by glucose during the stationary phase, *P. rettgeri* was grown in succinate alone and allowed to enter the stationary phase of growth, and additional carbon and nitrogen sources were added (Fig. 3). When glucose was added, alone (Fig. 3B) or together with ammonia (Fig. 3C), an increase in acylase specific activity was observed. This increase in activity was not observed when either succinate was added alone (Fig. 3A) or glucose and succinate were added together (Fig. 3D).

Addition of either citrate or glycerol during the stationary phase of cells grown in succinate caused the same dramatic increase of acylase activity as did glucose. This increase in acylase activity was prevented when 100 μg of chloramphenicol per ml was added together with citrate (Fig. 4).

Although this *P. rettgeri* strain cannot grow on glucose, mutants able to grow with this sugar as their sole source of carbon and energy were found at a very high frequency (10^{-3} to 10^{-4}). Mutants were isolated by simply plating 4×10^5 cells of this wild-type strain onto minimal medium containing glucose as the only source of carbon. One glucose-growing mutant (Glu-1) was compared with the wild type. With the exception of glucose, growth rates were found to be essentially the same for both strains when citrate, glycerol, pyruvate, or succinate was used as sole carbon and energy source. Neither strain grew in acetate or lactate. Like the wild type, the Glu-1 mutant expressed basal levels of acylase activity when grown in succinate minimal medium. Also like the wild type, the mutant expressed increased levels of acylase activity (~threefold) when the succinate minimal medium was supplemented with 0.2% glucose.

The formation of acylase was also monitored on the Glu-1 mutant and the wild-type strain while growing, respectively, in glucose and glyc-

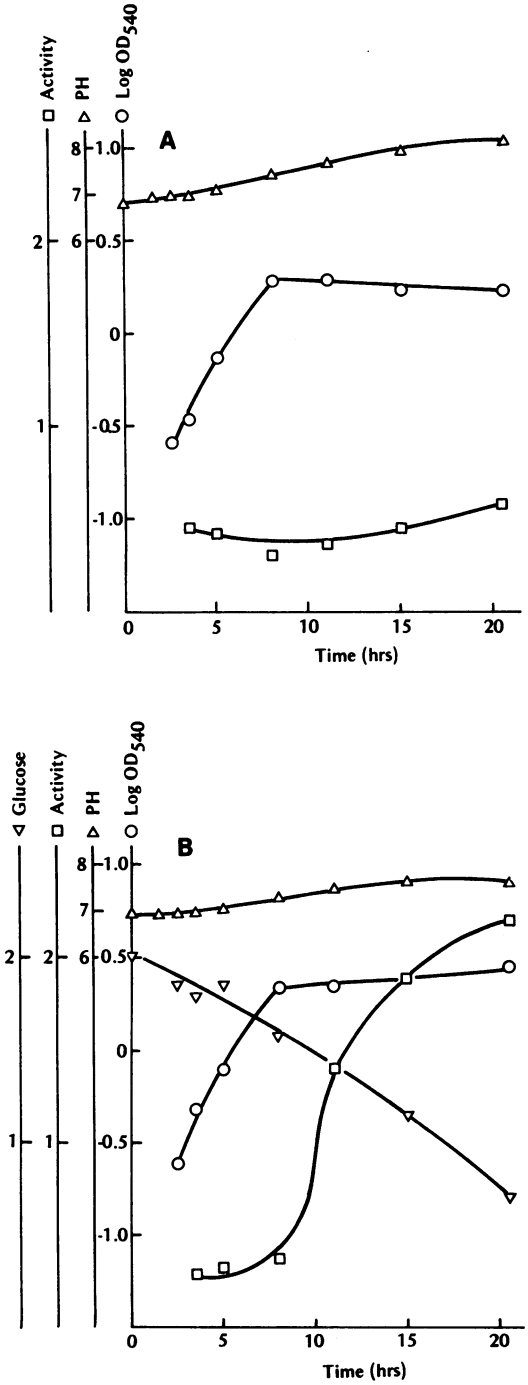

FIG. 2. Specific activity of *P. rettgeri* acylase during growth in succinate (A) and succinate supplemented with glucose (B). OD_{540}, Optical density at 540 nm.

erol as their only source of carbon and energy. The enzyme pattern observed was completely different from that observed in succinate-grown cells. When the mutant was grown in glucose and the wild-type was grown in glycerol, both strains expressed, during their logarithmic and stationary growth phases, three- to sevenfold higher levels of acylase activity than when grown on succinate. Furthermore, addition of succinate after the cells had entered the stationary phase had no effect on the levels of acylase made.

To test what effect other forms of carbon had on the formation of the acylase, the wild-type strain was grown on different growth substrates, and the specific activity of cellular extracts was measured (Table 2). Cellular extracts prepared from cells grown in succinate, fumarate, or malate contained basal levels of enzyme activity. On the other hand, extracts prepared from cells grown in citrate, pyruvate, glycerol, aspartate, glutamate, or α-ketoglutarate contained 3- to 15-fold increased levels of enzyme activity. There was no correlation between the efficiency by which the organism used a specific substrate for growth and the amount of enzyme made.

DISCUSSION

Although penicillin acylase is commercially important, its physiological role, primarily in non-penicillin-producing organisms, is not known. Attempts to define its physiological role have been hampered by the narrow substrate specificity observed in some acylases which have been purified to homogeneity and the vast and apparently unrelated distribution of this

TABLE 2. Effect of growth substrate on expression of *P. rettgeri* acylase

Carbon source (0.2%)[a]	Generation time (min)[b]	Sp act (μmol of product formed/h per mg of protein)[c]
Fumarate	83	0.3
Succinate + glucose	105	2.1
Pyruvate	109	1.1
Succinate	163	0.5
Glycerol	205	1.3
Glucose[d]	205	1.5
Citrate	206	2.6
Glutamate	584	2.2
Malate	603	0.3
Aspartate	659	5.7
α-Ketoglutarate		4.6

[a] Wild type strain was grown for 24 to 72 h in standard mineral base medium containing the carbon source indicated.

[b] Generation times were calculated turbidimetrically during the logarithmic phase of growth.

[c] Specific activity was determined in crude extracts as described in footnote *c*, Table 1.

[d] Glu-1 mutant was used.

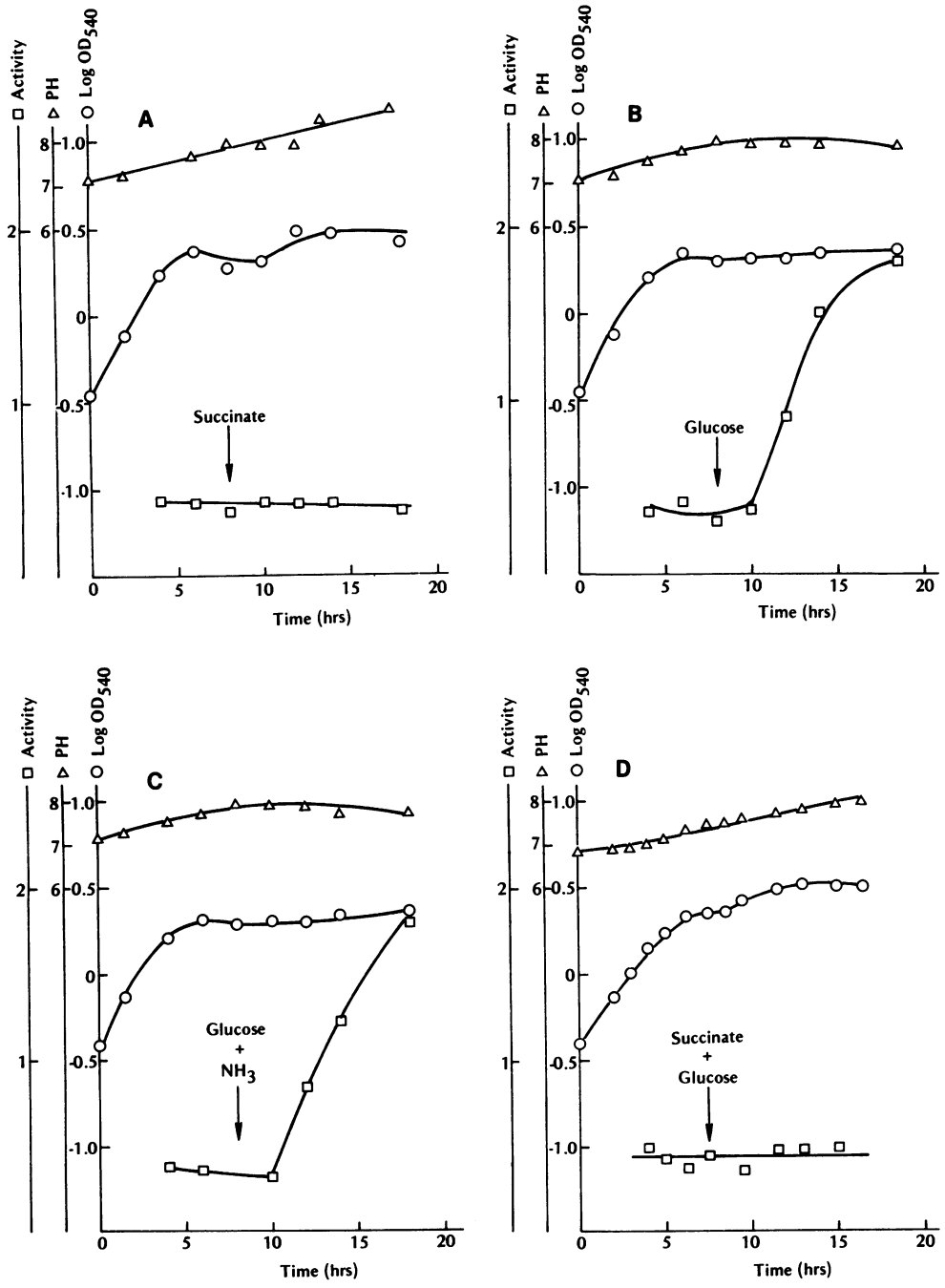

FIG. 3. Specific activity of *P. rettgeri* acylase during growth in succinate followed by the addition of succinate (A), glucose (B), glucose plus ammonia (C), and glucose plus succinate (D) as indicated. OD_{540}, Optical density at 540 nm.

activity throughout the microbial world (17). Not many microbes make penicillin acylases, but this enzymatic activity has been found in many different genera of molds, yeasts, and bacteria (17). Consequently, at one time or another, various physiological roles, ranging from conferring penicillin resistance to the organism to an incidental activity attributed to some structural protein, have been bestowed on this enzyme (9).

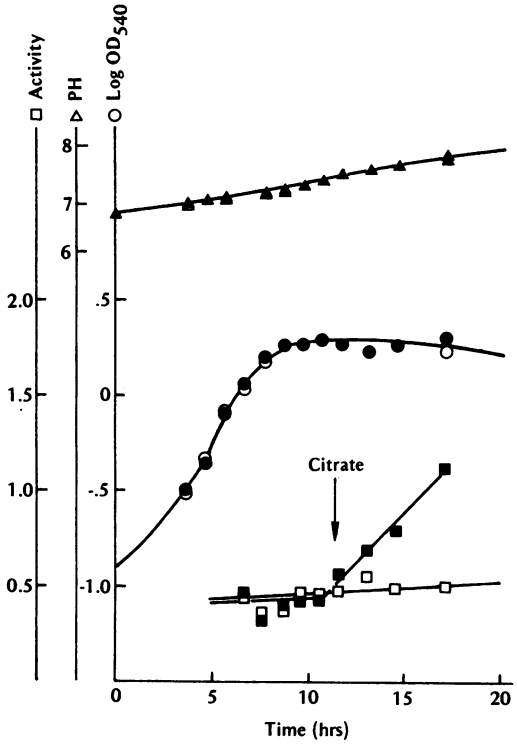

FIG. 4. Specific activity of *P. rettgeri* acylase during growth in succinate followed by the addition of citrate with (open symbols) and without (closed symbols) chloramphenicol. OD_{540}, Optical density at 540 nm.

Escherichia and *Proteus* are both enteric microbes and tend to occupy similar environmental niches. The two strains used in this study are unique to their genera in that they produce penicillin acylase. Both strains appear to produce similar levels of enzymes, but their respective acylases have different electrophoretic mobility in polyacrylamide gels (unpublished data). This suggests that the two strains may have evolved structurally different enzymes. In addition, these strains also appear to have evolved different mechanisms to regulate the production of their respective penicillin acylases. The Pen G acylase from *E. coli* ATCC 9637 and its auxotrophic mutant *E. coli* ATCC 11105 (2) has been, so far, the most intensively studied. Production of *E. coli* acylase decreases with increased oxygen tension (18) and growth temperature (10, 13, 16). Very low levels of acylase activity are detected in cells grown at 37°C or above. This is not a property of the acylase itself as the enzyme operates at an optimum temperature of 50 to 52°C (1). In addition to being regulated by oxygen levels and growth temperature, the *E. coli* acylase is induced by PAA, one of the products of the reaction, and is also subject to catabolite repression by glucose (10, 13, 16, 18, 19).

In sharp contrast to the penicillin acylase in *E. coli*, the *P. rettgeri* enzyme was not subject to temperature regulation and was neither induced by PAA nor catabolite repressed by glucose. The *P. rettgeri* enzyme appeared to be susceptible to repression by succinate but not by ammonia. The *P. rettgeri* acylase activity of crude extracts was not inhibited by succinate upon prolonged incubations, and crude extracts prepared from succinate-grown cells did not contain any inhibitory activity against the enzyme. When grown in succinate, *P. rettgeri* cells expressed very low levels of acylase activity during its entire growth phase. However, addition of energy-yielding compounds such as glucose, citrate, or glycerol during the stationary growth phase caused a dramatic increase in enzyme activity. This increase was prevented by chloramphenicol, which suggests that de novo protein synthesis was required for enzyme expression. Unlike ammonia, but like chloramphenicol, succinate prevented enzyme expression when added in conjunction with glucose at the stationary phase of growth.

Succinate repressed acylase expression even when the cells were supplemented with glucose in the growth medium. Acylase expression in glucose-supplemented medium took place only during the stationary phase of growth. During that time the levels of glucose were still relatively high (0.1%), whereas the levels of succinate were probably too low for effective repression.

Evidence that the production of acylase was not unique to the stationary growth phase was obtained by growing the wild type in glycerol and the Glu-1 mutant in glucose. During its logarithmic growth phase, when grown in glycerol, the wild type expressed sevenfold higher levels of acylase activity than when grown in succinate. While growing in glucose, the mutant strain expressed a threefold increase in acylase activity throughout the entire growth phase. Succinate had no effect on the specific activity of whole cells once the enzyme had been expressed at these higher levels.

The molecular mechanism for repression by succinate is still obscure. In *Pseudomonas*, in general, compounds which support high growth rates produce most repression (3, 12). No such correlation could be established for the repression of the acylase in *P. rettgeri*. In this organism succinate, fumarate, and malate seem to cause rather specific repression of the acylase. These dicarboxylic acids are not only intimately related metabolically, but also, in *E. coli* (14) and in a variety of other species (4, 5, 7), they share the same active transport system. Other sources of carbon, such as citrate, α-ketoglutar-

ate, aspartate, and glutamate, which can also be metabolically related to the C_4 -dicarboxylic acids via the tricarboxylic acid cycle, did not repress the acylase.

We are in the process of investigating this apparent specific repression of the acylase by C_4-dicarboxylic acids. We wish to know whether they can repress other enzyme systems in *P. rettgeri*. We also wish to know whether the three dicarboxylic acids share the same molecular mechanisms for acylase repression via either their potentially common transport system or facile metabolic interconversion. We feel that, even though the physiological role of penicillin acylase remains unknown, this strain of *P. rettgeri* could offer a microbial system, perhaps simpler than *Pseudomonas*, for studying repression affected by growth substrates other than glucose.

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