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Experimental Evolution of Penicillin G Acylases from *Escherichia coli* and *Proteus rettgeri*

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***Proteus rettgeri* and *Escherichia coli* W were shown to express structurally different penicillin G acylases. The enzymes had similar substrate specificity but differed in molecular weight, isoelectric point, and electrophoretic mobility in polyacrylamide gels and did not antigenically cross-react. When the organisms were subjected to environmental conditions which made expression of this enzyme essential for growth, spontaneous mutants were isolated that used different amides as the only source of nitrogen. These mutants acquired the ability to use amides for growth by deregulating the penicillin G acylase and by their evolution to novel substrate specificities. The enzymes expressed by mutants isolated from each genus appeared to have evolved in parallel since each acylase attained similar new substrate specificities when the organisms were subjected to identical selection pressure.**

Penicillin acylase (penicillin amidase [penicillin amidohydrolase], EC 3.5.1.11) is the enzyme used commercially to hydrolyze penicillin G to phenylacetic acid and 6-aminopenicillanic acid (6-APA), the intermediate used for the synthesis of many semisynthetic penicillin derivatives (Fig. 1). This acylase is produced by a wide variety of bacteria as well as fungi (26), but the physiological role of the enzyme is not known (25, 26).

Escherichia coli W (ATCC 9637) and *Proteus rettgeri* ATCC 31052 are among the microorganisms which produce penicillin acylase (25, 26). The regulation of the enzyme in *E. coli* W has been studied primarily in two strains, ATCC 9637 and a methionine auxotroph derived from this strain, ATCC 11105 (3). The acylase is induced by phenylacetic acid and subject to catabolite repression by glucose (11, 16, 19, 24, 27, 28). Regulation of this enyzme in *E. coli* is also affected by growth temperature. Maximal levels of penicillin acylase activity can be found in cells grown at 29°C or below (16, 20, 24), whereas only low levels of activity are detected in cells grown at 37°C. This low acylase activity is due to a decrease in the amount of acylase protein produced, since the enzyme itself is very active when assayed at temperatures above 37°C (2). The specificity of the enzyme produced by strains of *E. coli* is directed primarily to the phenylacetyl side chain of the penicillin molecule. This conclusion is based on the observation that the pure enzyme can hydrolyze a wide variety of phenylacetyl derivatives, such as phenylacetamide, phenylacetyl-L-amino acids, and the N-(3-carboxy-4-nitrophenyl) (NCN) derivative of phenylacetamide (18).

Regulation of the penicillin G acylase of *P. rettgeri* is different from that of the *E. coli* enzyme. Acylase production in *P. rettgeri* is not subject to temperature regulation, is expressed constitutively, and is repressed by the C_4 dicarboxylic acid intermediates of the tricarboxylic acid cycle, succinate, fumarate, and malate (8). Early experiments (15) with toluene-treated, freeze-dried cells suggested that the specificity of the penicillin G acylase in *P. rettgeri* is also directed towards the side chain of the penicillin molecule, since replacing 6-APA with 7-aminocephalosporanic acid has only a minor effect on enzyme activity (15). These toluene-treated cells were also capable of deacylating alkyl penicillins such as hexanoyl and heptanoyl derivatives of 6-APA (15).

The objective of this study was to apply experimental evolution techniques (4) in an attempt to alter the specificity of this commercially useful biocatalyst from each of these organisms. In this communication we describe a positive selection procedure, similar to one described in the patent literature (V. Vojtisek, J. Slezak, and K. Culik, Czechoslovak Socialist Republic patent 162,274, March 1973), which takes advantage of the amidase activity of the penicillin G acylase to yield spontaneous mutants with altered acylase properties. The mutants expressed deregulated levels of acylase and produced acylases with modified substrate specificity for the side chain of the penicillin molecule.

MATERIALS AND METHODS

Microbiological procedures. (i) Media. *P. rettgeri* ATCC 31052, *E. coli* ATCC 9637, and mutants derived from them were grown in enriched, minimal, or selective medium. The enriched medium was made up of 0.5% yeast extract, 1% tryptone, and 0.02 mg of thymine per ml in distilled water. The minimal medium used was Stanier basal salts (23) supplemented with 0.2% of either succinate (SMM), citrate (CMM), glycerol, or glucose as the carbon source. When a nitrogen source other than ammonia was used (selective media), all salts in the basal medium which had NH_4^+ ions were replaced with the corresponding salts containing either Na^+ or K^+ ions. Salts containing NO_3^- were replaced with Cl^- ions. The *P. rettgeri* selective medium contained succinate as the carbon source and 15 mM phenylacetamide, hexanamide (HEX), 6-bromohexanamide (BrHEX), or [1-(4-hydroxy-1-cyclohexenyl)]-acetamide (CYC) as the only nitrogen source. The carbon and nitrogen sources used in the selective media for *E. coli* were as follows. Selective medium 1 (derepression medium) contained 0.33% glucose, 15 mM phenylacetamide, and 0.03% phenylacetic acid inducer; selective medium 2 (constitutive medium) contained 0.33% glycerol and 15 mM phenylacetamide. To select mutants which were both derepressed and constitutive, selective medium 3 (phenylacetamide-glucose medium) was used. This medium was the

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

same as derepression medium except that no inducer was added. Selective medium 4 (HEX-glucose medium) and selective medium 5 (BrHEX-glucose medium) contained 0.33% glucose and 15 mM of the appropriate amide, respectively.

(ii) Mutant isolation. Spontaneously generated mutants were isolated after sequential transfers of the parental strains in the appropriate selective media. Growth-limiting amounts of inorganic nitrogen were added to the *P. rettgeri* selective medium during the initial transfers. Once the culture exhibited substantial growth in the selective medium without inorganic nitrogen, the concentration of succinate was increased to 1% and the concentration of the amide was gradually decreased to 0.02%. When substantial turbidity (~0.5 OD_{540} units) was observed after overnight growth in selective medium, cells were plated, and mutants from both genera were recovered by single-colony isolation. Their biochemical phenotype, growth, and level of acylase were compared with those of the parental strains. Growth rates were calculated from the logarithmic portion of the growth phase turbidometrically at 540 nm. A General Diagnostics Micro-ID kit test (Warner-Lambert Co., Morris Plains, N.J.) containing 15 biochemical tests for differentiation of the *Enterobacteriaceae* was used to compare the phenotypes of the wild-type and mutant strains.

Substrates and analytical procedures. (i) Synthesis of primary amides. The amides used in this study and their assigned numbers are presented in Table 6. Amides 2, 4, and 12 were commercially available. The rest of the amides were prepared by one of us (F.J.V.).

(ii) HPLC enzyme assay. Phenylacetic acid, the hydrolytic product of both penicillin G and phenylacetamide, was measured by high-performance liquid chromatography (HPLC) at 254 nm on a reverse-phase C_{18} column (Waters Associates) (250- by 4-mm inner diameter). The mobile phase consisted of 1 volume of 50 mM acetic acid and 1 volume of methanol. HPLC was also used to measure the hydrolysis of 7-aminodeacetoxycephalosporanic acid (7-ADCA) derivatives. For these determinations, a μNH_2 column (300- by 3.9-mm inner diameter; Water Associates) was used with a mobile phase containing water-acetonitrile-methanol-acetic acid in a ratio of 600:24:10:3. In whole cells, the acylase and amidase activities were measured after the washed cells were incubated at 37°C in 50 mM potassium phosphate buffer, pH 7.5, which contained either 15 mM penicillin G or 15 mM phenylacetamide. Before quantitative analysis by HPLC, the cells were removed by centrifugation, and the clear fluid was filtered through a membrane filter (0.45-μm pore size; Millipore Corp.). In cell extracts and purified enzyme preparations, the enzymatic activities were measured in the same way as with whole cells except that 1 volume of methanol was added to the reaction mixture before HPLC analysis.

(iii) Spectrophotometric enzyme assays. Acylase activity in cell-free systems was also measured with the NCN derivatives of phenylacetamide, HEX, BrHEX, and methyladipamate. The NCN derivative substrates were synthesized by the procedure described previously (8, 18). The 2-nitro-5-aminobenzoic acid formed after enzymatic hydrolysis of these substrates was followed kinetically at 405 nm (18). Amidase activity was measured kinetically at pH 7. The release of ammonia was coupled to the reductive amination of 2-oxoglutarate by glutamic dehydrogenase in a reaction mixture similar to that described by Kun and Kearney (17). Alkaline phosphatase and glutamic dehydrogenase activities were measured spectrophotometrically by the procedures described by Bergmeyer et al. (1) and Schmidt (22), respectively.

To measure the activity of the penicillin G acylase bound to immunoaffinity columns, 10 mM solutions of the substrates were recycled through the columns for a specific amount of time. Hydrolysis of amides and NCN and 7-ADCA derivatives was quantitated by the release of ammonia (17), 2-nitro-5-aminobenzoic acid, and 7-ADCA, respectively.

Kinetic analysis of purified soluble acylases was performed with the NCN derivatives. K_m (millimolar) and V_{max} (units per milligram of protein) were calculated from slopes and intercepts obtained from the Lineweaver-Burk plots of initial velocities at various substrate concentrations. Replots described by Cornish-Bowden (7) and Cleland (6) were used to determine the type of inhibition and inhibition constants (K_i) of the amides.

(iv) Definition of specific activity. Specific activities obtained in whole cells and cell-free preparations are expressed in units of enzyme activity (micromoles of substrate per minute) per milligram (dry weight) of cells or per milligram of soluble protein. Cell dry weight was calculated turbidometrically from a standard curve which plotted dry weight against turbidity. Protein was determined by the spectrophotometric method of Groves et al. (12), with bovine serum albumin used as the protein standard.

(v) Preparation of *E. coli* cellular fractions and acylase purification. To prepare *E. coli* periplasmic and cytoplasmic cellular fractions, a procedure similar to the one described by Oliver and Beckwith (21) was followed. All initial purification steps were done at 4°C, unless otherwise indicated. *E. coli* and *P. rettgeri* crude cell extracts were prepared from washed cells which were suspended in 50 mM potassium phosphate buffer, pH 7.5, and subjected to sonic disruption. Unbroken cells and cell debris were removed by centrifugation for 30 min at 30,000 × *g*.

To the clear supernatant fluid, protamine sulfate (0.25 mg/mg of protein) was added from a freshly prepared 4% stock solution made up in 50 mM potassium phosphate buffer, pH 7.5. The extract was stirred for 30 min, and the precipitate formed was removed by centrifugation at 30,000 × *g*. The supernatant fluid was diluted to 10 mg of protein per ml and brought to 40% saturation with ammonium sulfate. The 0 to 40% ammonium sulfate precipitate was removed by centrifugation (30,000 × *g*), and the supernatant was brought to 60% saturation with ammonium sulfate. The precipitate was recovered by centrifugation, dissolved in 10 mM potassium phosphate buffer, pH 7.5, and dialyzed overnight. The ammonium sulfate-precipitated *P. rettgeri* enzyme was dialyzed against the same buffer, but the *E. coli* enzyme was dialyzed against 1 mM Tris-hydrochloride, pH

8, containing 40 mM KCl. All subsequent purification steps were done at room temperature. Both dialyzed enzyme preparations were subjected to Cellex T anion exchange chromatography (Bio-Rad Laboratories, Richmond, Calif.). The *E. coli* enzyme was eluted during the column wash. The *P. rettgeri* acylase bound to the column and was eluted with a linear KCl gradient (0 to 0.2 M).

The *E. coli* enzyme was further purified on Sephadex G-200 equilibrated with 10 mM potassium phosphate buffer, pH 7.5. The peak containing the activity was pooled, concentrated, dialyzed against 50 mM sodium acetate, pH 5.5, and subjected to column chromatography (carboxymethyl-Sephadex). The *E. coli* acylase was eluted with a linear gradient of 0.1 to 0.3 M sodium acetate, pH 5.

(vi) Isoelectric focusing and gel electrophoresis. The isoelectric point of the purified enzymes was determined by the PAG plate procedure with a 3.5 to 9.5 pH gradient as recommended by LKB Instruments, Bromma, Sweden. Native and urea-denatured enzymes were analyzed on polyacrylamide gels with or without 6 M urea by the procedure of Davis (9). Molecular weights (MWs) were determined on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels by the procedure of Laemmli (18a), with protein MW standards from Bethesda Research Laboratories, Bethesda, Md.). Analyses of total soluble SDS-treated protein were done on 5 to 13% polyacrylamide gradient gels. The percent acylase in crude cellular extracts was determined by densitometry of the protein-stained gels.

(vii) Immunodiffusion assay and preparation of affinity column. Antiserum cross-reactivity was determined by the double immunodiffusion (Ouchterlony) technique with premade agar plates (Hyland Diagnostics, Deerfield, Ill.). Precipitin bands were stained with Coomassie blue G250. To prepare the affinity columns, antiacylase immunoglobulin G (IgG) was purified on Affi-Gel Blue (Bio-Rad). The purified IgG was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) in 0.15 M potassium phosphate buffer, pH 6.8. The acylase was bound to the immunoabsorbent by cycling excess acylase for five passages through the column bed. The column bed had been previously equilibrated with a solution of 0.5 M NaCl in 50 mM potassium phosphate buffer, pH 7.2. Before equilibration and enzyme loading, the columns were thoroughly washed with 6 M urea in the same buffer.

RESULTS

Development of positive selection protocol. The development of a positive selection procedure for penicillin G acylase in both *E. coli* and *P. rettgeri* was based on the ability of the acylase to hydrolyze amides. Both genera had phenylacetamidase activity. In *P. rettgeri* both the acylase and amidase activities copurified and were irreversibly inhibited by phenylmethylsulfonyl fluoride, and the acylase reaction was competitively inhibited by phenylacetamide (K_i, 0.1 mM). Phenylacetamide utilization in both genera was subject to the same regulation which controlled expression of the acylase, i.e., growth temperature, induction by phenylacetic acid, and repression by glucose in *E. coli* and by succinate in *P. rettgeri*. These observations indicated that the presence of penicillin G acylase conferred on each organism the ability to grow in phenylacetamide as the only nitrogen source and suggested that the same positive selection scheme could be applied to both organisms.

Mutant isolation from *E. coli* and degree of penicillin G deregulation. Initial attempts to isolate *E. coli* mutants which would grow directly in phenylacetamide-glucose medium were unsuccessful. This medium was apparently too selective, as the mutant sought would have to be both constitutive and catabolite derepressed for acylase expression. However, after a month of sequential transfer at 29°C in either constitutive (glycerol-phenylacetamide) or derepression (glucose-phenylacetamide-phenylacetic acid) medium, two spontaneously generated mutants were isolated and designated M2 and M3. These mutants expressed higher levels of acylase activity than the wild type when grown without inducer (ammonia-succinate) or under conditions of catabolite repression (ammonia-glucose-phenylacetic acid).

Both the constitutive (M3) and catabolite repression-independent (M3) mutants yielded double mutants (M42 and M43, respectively) after sequential transfers for several weeks in phenylacetamide-glucose medium. These double mutants (constitutive and derepressed) served as parental strains for the isolation, by the same sequential procedure, of subsequent strains which grew in phenylacetamide-glucose medium at higher temperatures (M42-1, M43-1, and M43-2) and in HEX (CapA) or BrHEX (BroA) as the only source of nitrogen.

The degree of deregulation exhibited by the *E. coli* mutants was determined from specific activity measurements in cells grown under different conditions (Table 1). All mutants expressed higher levels of acylase activity than the wild type regardless of the growth conditions. The ratio of acylase activity obtained with and without an appropriate carbon source causing catabolite repression and with and without inducer was calculated as a measure of relief from catabolite repression or constitutivity (Table 1). By this criterion, the CapA and BroA mutant strains were not catabolite repressed, and both were constitutive for acylase expression (theoretical ratio of 1). However, all strains secreted greater than 90% of their acylases into the periplasm; and based on specific activity and densitometry measurements of these periplasmic fractions on SDS gels, the BroA strain produced less acylase than its parental CapA strain.

Mutant isolation from *P. rettgeri* and degree of penicillin G deregulation. Spontaneously generated mutants that were resistant to repression were also isolated from the *P. rettgeri* wild type by the sequential transfer procedure. However, since the strain had been shown to deacylate hexanoyl

TABLE 1. Degree of penicillin G acylase deregulation in *E. coli* mutants

Strain	Parent	Sp act (10^2 μmol/min per mg of protein) in growth medium[a]:			Activity ratio[b]	
		A	B	C	Repression (B/C)	Constitutivity (B/A)
Wild type		0.03	1.38	0.05	27.6	46.0
M42	M2	1.18	15.53	5.83	2.6	8.2
M42-1	M42	10.87	12.53	7.55	1.7	1.2
CapA	M42-1	20.20	17.75	23.33	0.8	0.9
BroA	CapA	5.07	4.67	6.95	0.7	0.9
M43	M3	2.63	2.95	0.88	3.4	1.1
M43-1	M43	14.30	16.50	7.03	2.3	1.2
M43-2	M43-1	16.02	11.00	6.68	1.6	0.7

[a] Specific activities were determined in cell extracts with NCN-phenylacetamide as the substrate. Cell extracts were prepared from cells grown in SMM (A), SMM supplemented with 0.03% phenylacetic acid (B), or glucose minimal medium containing 0.03% phenylacetic acid (C).

[b] The specific activity ratio B/C measures the degree of catabolite repression, and B/A measures the degree of constitutivity. A ratio of 1 represents full constitutivity or catabolite derepression.

TABLE 2. Characterization of *P. rettgeri* mutants

Strain	Parent	Selection amide	Generation time (h) with nitrogen source[a]:					Acylase activity[b]		
			AS	PAM	HEX	BrHEX	CYC	Sp act (μmol/ min per mg of protein)	% Acylase	Activity ratio (CMM/SMM)
Wild type		None	2.3	16.2	NG	NG	NG	0.03	2	8
Capll	Capl	HEX	2.7	3.8	3.6	NG	NG	1.16	9	2
Cycl	Capll	CYC	2.2	3.2	4.8	NG	3.2	0.93	7	1.5
Brol	Capll	BrHEX	2.3	3.2	3.2	6.1	NG	0.53	5	1.2

[a] AS, Ammonium sulfate; PAM, phenylacetamide. The growth media contained 0.2% citrate and 15 mM of the compound indicated as the only nitrogen source. NG, No growth.

[b] Specific acylase activity was measured with NCN-phenylacetamide in crude cellular extracts from cells grown in CMM. For percent acylase determination, protein extracts (50 μg) from each strain were resolved by SDS gradient polyacrylamide gel electrophoresis. Purified acylase was used to locate the acylase protein bands which were well resolved from the rest of the soluble proteins. The percent acylase was calculated by densitometry of the Coomassie blue-stained protein bands. Specific activity ratios were determined for extracts prepared from cells grown in CMM and SMM.

penicillins (15), and to apply maximal selective pressure during the serial transfers, HEX, a weaker competitive inhibitor (K_i, ~30 mM) than phenylacetamide, was used as the only source of nitrogen.

A mutant was isolated (Capl) which grew in HEX as the only source of nitrogen. Although the strain grew poorly in the selective medium, cellular extracts had approximately ninefold higher levels of acylase activity than the wild type. Further sequential transfers of Capl in the same medium yielded a second mutant (Capll) which grew well in HEX. Capll served as the parental strain for further selection on either BrHEX or CYC. Mutants Brol and Cycl were isolated from the BrHEX and CYC selective media, respectively.

Unlike the wild-type and parental strains, each *P. rettgeri* mutant grew with the selection amide as the only nitrogen source and was highly resistant to succinate repression of acylase expression (Table 2). Whereas the ratio of acylase activity (derepressed [CMM]/repressed [SMM]) was 8 for the wild type, it was much lower for the mutant strains (Table 2). As with the *E. coli* strains, a ratio of 1 would represent complete derepression.

All mutants produced higher levels of acylase than the wild type did when grown in either SMM (repressive carbon source) or CMM (nonrepressive carbon source). However, both the Cycl and Brol strains produced less acylase than their parental strain, Capll (Table 2).

Evidence for experimental evolution of penicillin G acylases. The acquired ability of the *E. coli* BroA strain and *P. rettgeri* Brol and Cycl strains to use their respective selection amide for growth was not the result of higher levels of acylase expression than their parents had. Although the parental strains *E. coli* CapA (Table 1) and *P. rettgeri* Capll (Table 2) produced more acylase protein, they failed to grow in the selection substrate. Therefore, the progeny strains had either evolved an alternative pathway to catabolize the amides or were expressing acylases with a different substrate specificity.

To compare the catalytic differences between the acylases, the enzymes from the *P. rettgeri* mutants Capll, Brol, and Cycl and the *E. coli* mutant BroA were purified. Based on polyacrylamide gel electrophoresis with and without SDS, the enzymes were at least 95% pure. For a convenient purification procedure for all the acylases produced by the different strains, purified IgG from rabbit antiserum prepared against the *P. rettgeri* Brol and *E. coli* BroA strain enzymes was bound to a cyanogen bromide-activated Sepharose 4B resin. The enzymes could be retained on the columns containing specific antiserum, and inactive protein could be eluted with 6 M urea. The inactive preparations could be partially reactivated by overnight dialysis against 50 mM potassium phosphate buffer, pH 7.5 (*E. coli*, ~30%; *P. rettgeri*, ~50%). The material eluted from the immunoabsorbent columns gave a single band in native polyacrylamide electrophoresis coincidental with acylase activity and, in polyacrylamide gels containing SDS, the same heavy and light chains corresponding to the acylase. The low recovery of activity after elution with 6 M urea prevented us from using these columns to purify the acylases from the various strains in soluble form to compare them catalytically. However, the enzymes maintained over 90% of their activity while bound to the affinity columns, and the specific activity of the bound acylase was similar to that of the purified enzymes in solution. In addition, the columns bound the same number of units of enzyme per volume of resin whether crude or purified acylase preparations were used to load them, and they bound the acylases with equal efficiency. Since the columns appeared to bind acylase exclusively in an active conformation, they offered a convenient method for comparing in situ the catalytic differences between the enzymes produced by the various strains.

Cellular extracts prepared from the wild-type and mutant strains were used to load identical immunoaffinity columns prepared with anti-Brol or anti-BroA acylase antibody. The charged columns were assayed for amidase activity by recirculating 10 mM solutions of either HEX, CYC, or BrHEX, and the amount of ammonia released was measured.

The specific activities of the immobilized *P. rettgeri*

TABLE 3. Specific amidase activities of soluble and immobilized *P. rettgeri* penicillin G acylase

Enzyme source and substrate	Amidase sp act[a]			
	Wild type	Capll	Cycl	Brol
Immobilized				
HEX	0.11	1.13	0.31	1.10
CYC	0.51	0.15	1.85	0.07
BrHEX	<0.01	<0.01	<0.01	1.19
Soluble				
HEX	ND	1.17	0.31	1.18
CYC	ND	0.07	1.21	0.06
BrHEX	ND	0.07	0.03	1.04

[a] Amidase activities are expressed as micromoles of ammonia released per minute per milliliter of packed resin (bound acylase) or per milligram of protein (soluble acylase). ND, Not done.

acylases differed from each other with respect to the substrate (Table 3), and these differences correlated with growth on the selection amides (Table 2). The three mutant acylases, when compared in their purified soluble form, gave the same pattern of activity with the amides (Table 3).

A similar activity profile was observed with the 7-ADCA derivatives. Although all four immobilized enzymes had similar specific activities for the phenylacetyl derivatives, the highest rate of hydrolysis for the hexanoyl side chain was observed with the Cap11 strain acylase, and the highest rate of hydrolysis for the 6-bromohexanoyl side chain was observed with the Bro1 strain acylase. In all the *P. rettgeri* strains, the rate of hydrolysis for the 7-ADCA derivatives was lower than for the corresponding amides. The four acylases, when eluted from these columns with 6 M urea, each gave in SDS-polyacrylamide gel electrophoresis heavy and light chains of identical MWs, had the same relative electrophoretic mobilities in polyacrylamide gels, and had indistinguishable isoelectric points.

In contrast to the *P. rettgeri* enzymes, the acylase from the *E. coli* wild-type and deregulated mutant CapA strains had higher activity for the 7-ADCA derivative than for the corresponding amide regardless of the side chain tested (Table 4). However, this was not the case for the BroA mutant enzyme. Its activity was similar for the 7-ADCA derivatives and amides. The enzymes from the deregulated CapA mutant and wild-type strains had similar specific activities for all substrates tested. In contrast, the acylase derived from the BroA strain hydrolyzed the halogenated derivatives more effectively than either the wild-type or parental CapA strain enzyme did and had improved activity for HEX. When eluted from the columns, the three enzymes had the same relative electrophoretic mobility in their native form and the same heavy and light chains after SDS treatment (Fig. 2).

The kinetics of the three soluble purified *P. rettgeri* acylases were compared with available acylase model substrates (Table 5). The specificities (V_{max}/K_m) of the Cap11 and Bro1 strain enzymes were quite different. The *P. rettgeri* Bro1 enzyme's specificity for the BrHEX derivative was 70-fold greater than that of the parental enzyme and 58-fold greater than the enzyme from the Cyc1 sibling strain. The Bro1 strain enzyme exhibited a 50-fold increase in V_{max} for this substrate. These kinetic results support the interpretation that the differences in specific activity observed with the immobilized acylases reflected catalytic evolution of the enzymes. Although the specificities of the Cap11 and Cyc1 strain enzymes were the same for the substrates tested (Table 5), the Cyc1 acylase was also considered an evolved penicillin G acylase since the soluble and immobilized Cyc1 strain acylase hydrolyzed CYC at a rate 12 to 17 times higher than that of the parental Cap11 strain enzyme (Table 3). Neither the NCN nor the 7-ADCA derivatives for the cyclohexenoyl amide side chain were available to test the relative hydrolysis of these compounds by the acylases, but the results obtained with the other substrates indicated that amide hydrolysis correlated well with acylase activity. Therefore, the Cyc1 strain acylase should also be expected to hydrolyze the NCN and 7-ADCA derivatives more efficiently than the parental strain enzyme.

TABLE 4. Activities of *E. coli* acylase bound to immunoabsorbent columns[a]

Substrate	Strain	Acylase activity[b]			
		R = 7-ADCA		R = NH_2	
		Sp act	% Conversion	Sp act	% Conversion
$CH_3(CH_2)_4COR$	Wild type	2.57	8.2	0.88	5.6
	CapA	2.43	7.8	0.97	5.2
	BroA	2.88	9.2	2.70	14.6
$BrCH_2(CH_2)_4COR$	Wild type	0.75	2.4	0.03	0.75
	CapA	1.63	2.0	0.05	1.00
	BroA	2.75	8.8	2.47	26.20

[a] Three identical immunoabsorbent columns (0.4-ml packed-bed volume) were loaded with acylase from crude cellular extracts.

[b] Acylase activities for the 7-ADCA and amide derivatives were determined as described in the text. Activities are expressed as micromoles of 7-ADCA or ammonia released per minute per milliliter of bed volume. Percent conversion represents the amount of substrate which had been consumed when the enzymatic reaction was terminated.

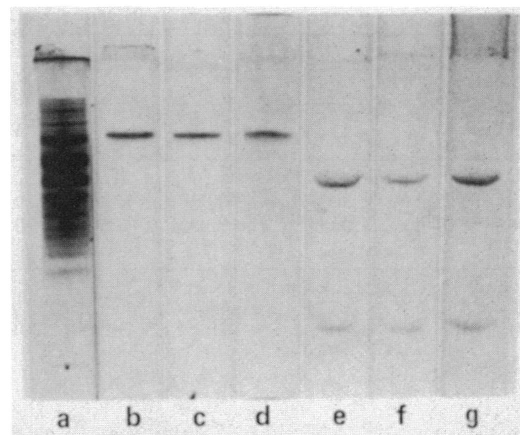

FIG. 2. Electrophoresis of affinity-purified *E. coli* wild-type and mutant acylases in native (lanes a–d) and denaturing (lanes e–g) polyacrylamide gels. Lanes: a, crude BroA; b and e, wild type; c and f, CapA; d and g, BroA.

Since only one of the *E. coli* enzymes was available in pure form, a specificity comparison via kinetic analysis could not be done. However, if the specific activity differences observed with the immobilized *E. coli* acylases reflect true differences in specificity, as with the *P. rettgeri* enzymes, then the acylase expressed by *E. coli* strain BroA should also be considered an evolved enzyme.

Evidence for parallel evolution of penicillin G acylase. The *E. coli* BroA and *P. rettgeri* Bro1 mutants produced acylases which were indistinguishable from those of their parental strains in terms of MW, isoelectric point, electrophoretic mobility, and cross-reactivity with specific antisera. They both also produced lower levels of acylase than their parental strains and grew in BrHEX primarily because they expressed a catalytically altered acylase.

However, the two organisms produced structurally different acylases. Each acylase reacted with antiserum raised against the acylase of its genus, but neither cross-reacted with the nonhomologous antiserum. The isoelectric point of the *E. coli* and *P. rettgeri* acylases was 7.4 and 5.5, respectively. In polyacrylamide gels under nondenaturing conditions, the *P. rettgeri* acylases migrated ahead of the *E. coli* enzyme (Fig. 3), and each enzyme dissociated into two distinct polypeptides when subjected to polyacrylamide electrophoresis with either urea or SDS (Fig. 3). In gels containing urea, the *E. coli* polypeptides migrated more slowly than their *P. rettgeri* counterparts. In gels containing SDS, the apparent MWs were determined to be 62,000 and

TABLE 5. Specificity of purified mutant acylases[a]

Substrate[b]	*P. rettgeri*									*E. coli* BroA		
	Cap11			Cyc1			Bro1					
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
NCNPA	0.02	12.9	645	0.02	12.3	615	0.05	13.5	270	0.03	9.2	307
NCNHA	0.32	0.17	0.53	0.40	0.04	0.10	0.40	0.35	0.90	0.16	0.37	2.3
NCNBR	0.40	0.004	0.001	0.50	0.006	0.01	0.31	0.23	0.70	0.22	0.11	0.50
NCNME							6.00	0.20	0.03	9.10	0.14	0.02

[a] Kinetic parameters were calculated as described in the text from initial velocities determined against different concentrations of the acylase model substrates at pH 7.5. Values for K_m and V_{max} are expressed as millimolar and as units per milligram of protein, respectively.
[b] NCNPA, NCN-phenylacetamide; NCNHA, NCN-HEX; NCNBR, NCN-BrHEX; NCNME, NCN-methyladipamate.

23,000 for the *E. coli* polypeptides and 66,000 and 24,500 for the *P. rettgeri* polypeptides. The difference in size between the heavy and light polypeptides was essentially the same for both enzymes (~40,000 daltons). Catalytically the acylases were very similar. Both soluble forms of the enzymes had a similar pH optimum for deacylation and the same specificities for the NCN derivative acylase model substrates tested (Table 5).

Both immobilized acylases also had similar relative amidase activities for the 11 amides tested, except CYC, and both enzymes were able to distinguish between the *cis* and *trans* isomers of 3-hexenamides (compounds 3 and 9, Table 6).

DISCUSSION

In this communication we describe an example of parallel evolution in the laboratory. In nature, *E. coli* ATCC 9637 and *P. rettgeri* ATCC 31052 have evolved different regulatory controls over production of their penicillin G acylases (8) and different proteins to catalyze the same hydrolytic reaction. The acylases differed in MW, isoelectric point, electrophoretic mobility, and antigenicity. We applied selective pressure for the penicillin G acylase to both organisms and found that they responded in the same fashion. In each case, the selection first favored organisms with deregulated levels of acylase, and after the enzyme was overproduced, further selection resulted in mutants which expressed lower levels of acylase than their parental strains but the enzymes had increased specificity for the selection substrate. Furthermore, after selection on BrHEX, each organism produced penicillin G acylases which had evolved to the same catalytic phenotype. The catalytically altered acylases produced by the *E. coli* BroA and *P. rettgeri* Brol mutants had the same specificity (V_{max}/K_m) for the phenylacetyl, 6-bromohexanoyl, and methyladipoyl acylase side chains. The enzymes also had similar relative amidase activity for most of the substrates tested. Neither enzyme was active on *trans*-2-hexenamide, and the activity of both enzymes for the *cis*-3-hexenamide olefin was substantially higher than for the corresponding *trans* isomer. No activity against adipamic acid was detected with either acylase. The enzymes had apparently similar hydrophobicity at the active site, since both were able to hydrolyze the less polar methylester derivative. The only major difference between the enzymes was observed in the hydrolysis of CYC. The *P. rettgeri* Brol strain acylase had lower activity than the *E. coli* BroA enzyme for CYC. Since these two enzymes were structurally different and evolved to the same catalytic extent when the organisms were subjected to identical selection pressure, we concluded that they had evolved in parallel and they represent, to our knowledge, the first example of parallel evolution accomplished in the laboratory.

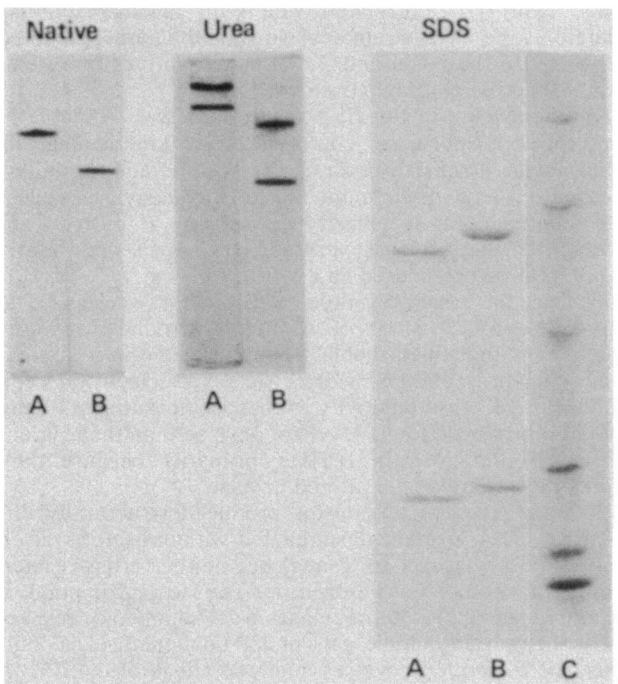

FIG. 3. Polyacrylamide gel electrophoresis of native and denatured *E. coli* strain BroA (lanes A) and *P. rettgeri* Brol (lanes B) acylases. Lane C, MW protein standards (Bethesda Research Laboratories). The relative electrophoretic mobility of the native enzyme was 0.22 and 0.35 for the *E. coli* and *P. rettgeri* acylases, respectively.

Other laboratories have used positive selection techniques to isolate mutants from various microbial sources which evolved to express enzymes with altered substrate specificities (5, 10, 13, 14, 29). Clarke (4) concluded that the rate-limiting step in the study of experimental evolution was the design of a suitable selection procedure. Although the physiological role of penicillin G acylases is unknown (25, 26), we were able to design a positive selection protocol for these enzymes because they had amidase activity. By forcing such catabolic function on the enzymes, we accomplished evolution in the laboratory. In this study, all strains subjected to amidase selection expressed deregulated levels of penicillin G acylase. The *E. coli* CapA and BroA mutants were fully constitutive and resistant to glucose catabolite repression. The resistance to catabolite repression was spe-

TABLE 6. Amidase activities of *E. coli* BroA and *P. rettgeri* Brol penicillin G acylases

Compound no.	Substrate	Relative amidase activity[a]	
	Structure	*E. coli* BroA	*P. rettgeri* Brol
1	CONH—(ring)—NO_2, COOH	1	1
2	(ring) $CONH_2$	36.2	49.3
3	$CONH_2$	9.9	16.6
4	(ring) O $CONH_2$	4.5	5.7
5	Br $CONH_2$	3.1	2.8
6	$CONH_2$	4.9	3.6
7	$CONH_2$	0.5	1.0
8	CH_3OOC $CONH_2$	0.4	0.4
9	$CONH_2$	0.5	0.4
10	HO—(ring) $CONH_2$	1.0	0.1
11	$CONH_2$	0.0	0.0
12	HOOC $CONH_2$	0.0	0.0

[a] Amidase activities were determined by measuring the release of ammonia by the immunoabsorbed bound enzymes and are expressed relative to the deacylation of the NCN-HEX model substrate (compound 1).

cific to the acylase gene since beta-galactosidase activity remained sensitive to repression (unpublished data). Mutations in the cyclic AMP proteins, adenylate cyclase, etc., would also be expected to affect the sensitivity of beta-galactosidase to catabolite repression. *P. rettgeri* is constitutive for acylase (8), and although the strains isolated were more resistant to succinate repression than the wild type, none of them was fully derepressed. The biochemical nature of succinate repression of the *P. rettgeri* acylase is currently not clear, and speculations on the nature of the mutation(s) which rendered the strains resistant to repression are premature. In addition to constitutivity and resistance to repression, the mutants from both genera were hyperproducers of the acylase. The amount of acylase protein expressed by all mutants was severalfold higher than that expressed by the wild type whether the strains were grown under induced or derepressed conditions or both. The acylase side chain specificity was altered only after the organisms became hyperproducers of the enzyme. To use a novel substrate for growth, the strains seemed first to take advantage of the minimal recognition of the enzymes for the growth-limiting substrate, and after further selection mutations which increased specificity for substrate recognition then became apparent. Although we did not test whether the *P. rettgeri* Capl strain expressed a more efficient acylase on HEX than the wild type did, it is likely that this strain, like the *E. coli* CapA strain, grew in this amide because of acylase overproduction rather than enzyme evolution. Both *P. rettgeri* Capl and *E. coli* CapA produced the same level of acylase activity. After further selection in either HEX or BrHEX, mutants from both genera (*P. rettgeri* Capll and *E. coli* BroA) were isolated which expressed catalytically modified acylases. Attempts to isolate mutants which grew in BrHEX were not successful with strains which produced low levels of acylase. These observations are consistent with those reported by Clarke (4), which suggest that overproduction may be a prerequisite for experimental evolution.

The mutation(s) responsible for enzyme hyperproduction is of commercial importance but has not been characterized. However, in *E. coli* it is known that the acylase in the wild type is under negative control, since cloning and expression of the acylase gene result in the production of constitutive strains (20). Consequently, a mutation in *E. coli* causing overproduction of the acylase by placing it under a more efficient positive control is highly improbable. The acylase genes from the wild types and several of the evolved strains from this study have been cloned, and we are in the process of characterizing at the molecular level the evolutionary mutations which affected the expression and specificity of the *E. coli* and *P. rettgeri* penicillin G acylases.

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