Isolation and Characterization of a Pseudomonas Strain Producing Glutaryl-7-Aminoccephalosporanic Acid Acylase

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Several screening methods were developed for the selection of Pseudomonas strains capable of hydrolyzing glutaryl-7-aminocephalosporanic acid to 7-aminocephalosporanic acid. An isolate exhibiting high acylase activity, designated BL072, was identified as a strain of Pseudomonas diminuta. It grew optimally at pH 7 to 8 and at a temperature of 32 to 40°C, but acylase activity was highest when the strain was grown at 28°C. Mutants of BL072 were generated by nitrosoguanidine treatment and screened for increased production of glutaryl-7-aminocephalosporanic acid acylase. A superior mutant gave a fourfold increase in acylase titer. The cell-associated acylase had similar activities against various glutaryl-cephembs but had undetectable activity against cephalosporin C. This acylase may prove useful for the conversion of cephalosporin C to 7-aminocephalosporanic acid.

Acylases which have the ability to hydrolyze penicillin G and penicillin V to the β-lactam nucleus 6-amino-penicillanic acid (6-APA) have been described in many reports (7, 10, 15–17, 24) and are widely used in industry to produce intermediates for the manufacture of semisynthetic penicillins and cephalosporins. As new generations of cephalosporin-based antibiotics supplant penicillins in medical treatment, the cephalosporin nucleus 7-aminocephalosporanic acid (7-ACA) has become a key manufacturing intermediate. Originally, it was hoped that an acylase as active as some of the penicillin acylases would be found to convert cephalosporin C directly to 7-ACA; however, acylases specific for the unusual D-aminoacidic side chain of cephalosporin C are rare. The cephalosporin C acylases which have been described (2, 4, 8, 12, 14, 23) generally have low activity and have not yet proven useful industrially.

Considerable research has centered on discovering acylases which are active upon cephalosporin C derivatives. The D-aminoacidipyl side chain of cephalosporin C can be oxidatively deaminated either chemically (28) or enzymatically by D-amino acid oxidase enzymes (5, 13, 26, 29, 30) to yield glutaryl-7-aminocephalosporanic acid (GI-ACA), as shown in Fig. 1. This derivative is more susceptible to specific enzymatic hydrolysis to form 7-ACA as judged by several reports in the literature (2, 11, 19, 20, 25). Although GI-ACA acylases are difficult to find in nature, their use in industrial production could yield significant cost reductions.

Several problems need to be overcome to discover new, useful GI-ACA acylases. Usually, bacterial isolates contain strong β-lactamase and esterase activities which destroy GI-ACA before it can be converted to the desired β-lactam nucleus. These activities would cause acylase-positive isolates to be missed in typical screening assays. The use of non-β-lactam, chromogen-generating screening compounds can eliminate these competition problems, but the acylases discovered from such a screening process are most often not active against GI-ACA. Compounds very closely related to GI-ACA are the most apt to be useful as screening tools. As a result, we found that glutaryl-desacetoxy ACA (GI-ADCA) (Fig. 1) was the most useful screening compound because of its resistance to competing enzymatic activity and its close relation to GI-ACA. We were able to develop quick and specific screening assays for GI-7ADCA acylase activity that allowed us to assay 100 isolates per day by high-performance liquid chromatography (HPLC) assay or 500 per day by a colorimetric method. Additionally, a refinement which determined the specific activities of mutant isolates allowed us to increase the specific activity of an original acylase strain.

MATERIALS AND METHODS

Chemicals. Cephalothin, cephalosporin C, 7-ACA, and 7-ADCA were obtained from Bristol-Myers Squibb Company. Glutaryl derivatives of 7-ACA and 7-ADCA were prepared by acylation with glutaric anhydride followed by subsequent recrystallization (25). Glutaryl-desacetyl-7-ACA (GI-deacetyl-ACA) was prepared from GI-ACA by reaction with an immobilized esterase.

Microorganisms. Soil isolates were obtained by plating diluted soil suspensions onto a Pseudomonas-selective medium (9). Individual colonies were streaked onto a plate medium containing glucose (1 g/liter), peptone (Difco) (10 g/liter), beef extract (BBL) (5 g/liter), yeast extract (FIDCO) (1 g/liter), sodium chloride (5 g/liter), glutaric acid (0.5 g/liter), and agar (18 g/liter). Streaks were grown for 3 days at 28°C. An agar plug (0.3 by 1.5 cm) was cut out from the streak and mixed with 0.5 ml of 0.1 M phosphate buffer (pH 7.0) for use in the enzyme activity screening assay. Stock cultures were stored as vegetative preparations frozen in seed medium (1% peptone and 0.5% Yeast Extract [pH 7.3]) at −70°C.

Liquid culture of BL072. The seed medium described above was used for starting cultures of BL072 from colonies or frozen vegetative preps before inoculation into acylase production medium. Seed medium was also used to study growth rates at various temperatures. Acylase production medium contained 1% NZ Amine HD (Sheffield), 0.5% yeast extract, 2% casein hydrolysate (Deltown Chemurgic CER90C), and 0.03% polypropylene glycol at pH 7.3. Small batch fermentations were carried out in 20 ml of medium in
a 125-ml shaken Erlenmeyer flask. When medium pH was varied, the stated pH was that of the production medium before sterilization. Typically, the pH of batch fermentations rose by 1.0 to 1.5 during the fermentation cycle. Tank cultivations performed in 2-liter stirred fermentors utilized the production medium described above and additionally contained sulfuric acid (pH 8.0) and polypropylene glycol antifoam additions as needed; air flow was at 0.7 vol/vol/min. The temperature was controlled at 28°C. Dry cell weight determinations were done by centrifuging broth, washing the cells with water, drying the pellets at 60°C under vacuum to constant dry weight, and weighing the resulting residue.

**Generation of BL072 mutants.** Using a procedure adapted from reference 1, 12 mg of N-methyl-N' nitro-N-nitroso- guanidine crystals were added to 25 ml of log-phase BL072 cells to generate mutants. After 10 min of agitation at 28°C, the suspension was diluted and plated onto seed medium agar and incubated for 2 days at 37°C.

**Enzyme incubations.** Agar plugs with soil isolates were combined with 1% solutions of GI-ADCA, GI-ACA, or cephalothin in 0.1 M Tris (pH 8.0). The reaction mixes were shaken at 37°C for 6 to 18 h.

Whole broth assays typically contained 0.5 ml of broth added to 0.5 ml of 2% GI-ADCA in 0.3 M Tris-Cl (pH 8.0). Broths were agitated for 3 h at 37°C. Reactions were stopped by the addition of 4 ml of 25 mM sulfuric acid, and the mixtures were centrifuged to give a clear supernatant at a pH of 2.0. The cepham products in this acidic supernatant were stable for more than 5 h.

Mutants of BL072 were screened with a procedure that measured cell mass and evaluated enzyme yield in terms of specific activity. Isolates were streaked on production medium agar and grown for 2 days at 28°C. The cell mass (2 to 5 mg) was scraped from the agar and weighed in a small test tube. A 0.5-ml volume of 1% GI-ADCA in 0.1 M Tris (pH 8.0) was added, and the reaction mixture was agitated at 37°C for 2 h. The reaction was stopped with 0.5 ml of 0.2 M sulfuric acid. Cell-free or heat-inactivated controls gave no conversion of the substrate to product.

**HPLC assay of products.** Cell-free supernatants from the centrifugation of reaction mixtures containing product representing 2 to 10% conversion of substrate were injected on an HPLC column as follows. Two microliters were injected onto a C18 reverse-phase HPLC column (4.2 by 50 mm) which had a mobile phase of 10 mM octylsulfonic acid–10% methanol–0.1% phosphoric acid flowing at 3 ml/min. Compounds were detected by A_{235}. Typical retention volumes for GI-ADCA and 7-ADCA were 1.8 and 4.2 ml, respectively. At a lower methanol concentration (7.5%), retention volumes for GI-desacetyl-ACA, desacetyl-ACA, Gl-ACA, and 7-ACA were 1.2, 1.8, 4.2, and 5.0 ml, respectively. The void volume was 0.8 ml.

Product formation was measured in micromoles per minute per milliliter of broth (international units per milliliter).

**Ninhydrin colorimetric assay of products.** A colorimetric method for the detection of 7-ACA and related compounds was adapted from the method of Marrelli (18). Reaction mixtures (0.1 ml) were added to wells of a microtitr dish, followed by the sequential addition of 0.05 ml of 2% citric acid in 0.8 N NaOH–formic acid (10/1) and 0.05 ml of 2.5% ninhydrin solution in 2-methoxyethanol. After 10 min of incubation at 25°C, the A_{415} and A_{530} of each well in the microtiter dish was determined by an automated spectrophotometer. The product concentration was estimated by the difference in absorbance (optical density [OD]) at these two wavelengths (ΔOD = OD_{415} - OD_{530}).

**Characterization and identification of isolates.** Taxonomic characteristics were investigated according to reference 6 and by using references 22 and 27 as definitive works.

**RESULTS AND DISCUSSION**

**Selective isolation and screening of pseudomonal bacteria.** Initial attempts to isolate microorganisms containing glutaryl-ACA acylase activity resulted in all positive strains being members of the genus *Pseudomonas*. Therefore, subsequent screening of soil isolates concentrated on this type of bacteria by using a *Pseudomonas*-enriching isolation medium (9). By using this method, 95% of the isolates generated showed the gram-negative, oxidase-positive properties typical of the genus *Pseudomonas*.

We tested several compounds, such as glutaryl-3-nitroanilide and glutarylcoumarin, with the goal of detecting GI-ACA acylase activity while circumventing substrate degradation by β-lactamases and esterases. However, isolates with positive acylase activity against these compounds did not prove to have authentic GI-ACA acylase activity because of differences in acylase specificity. Subsequent screening was done with the compounds cephalothin and Gl-ADCA, which we found to have reduced susceptibilities to β-lactamase and esterase but which were structurally similar to Gl-ACA.

Five thousand isolates were screened for activity against either cephalothin or Gl-ADCA by using a combination of a ninhydrin colorimetric screening assay and an HPLC screening assay to detect production of β-lactam nuclei. The ninhydrin method was less accurate than the HPLC method but allowed the screening of up to 500 isolates per day. A good correlation was observed between the absorbance...
difference (OD450 - OD630) and the concentration of product (7-ADCA or 7-ACA) in the concentration range of 50 to 500 μg/ml. Little interference was observed from growth media, amino acids, or reaction substrates. The HPLC method was used to screen 100 isolates per day and was more specific than the ninhydrin method. In addition to screening, the HPLC method was used to confirm positive isolates from the ninhydrin screening assay.

Seventeen cephalothin-positive colonies and six GI-ADCA-positive colonies were found. The cephalothin-positive cultures proved to be GI-ACA acylase negative and were not investigated further. The GI-ADCA acylase active isolates were all shown to have activity against GI-ACA but not against cephalosporin C. The culture with the best acylase properties and low β-lactamase activity, BL072, was selected for further study.

Characterization and identification of BL072. Strain BL072 was a short rod incapable of growing in chemically defined minimal medium and therefore was chemoheterotrophic. BL072 colonies are convex, do not produce any visible pigments, and lack the ability to hydrolyze starch and gelatin but accumulate poly-β-hydroxybutyrate intracellularly. The morphological, cultural, and physiological characteristics are as follows. Strain BL072 is gram negative; cells are straight or curved rods with rounded ends (0.5 to 1.0 by 1.5 to 2 μm). It is positive for motility and has one unipolar flagellum. It is negative for sporulation, will not grow on minimal medium, and forms circular, convex, smooth, and colorless colonies on nutrient medium. Strain BL072 is negative for soluble pigment production; yellow or orange soluble pigments; gelatin liquefaction; starch hydrolysis; denitrification, nitrate reductase, indole production; Voges-Proskauer reaction; acid production from xylose, glucose, fructose, sucrose, and lactose; and citrate utilization. BL072 is positive for organic growth factor requirements, oxidation reaction, catalase, and poly-β-hydroxybutyrate accumulation. The temperature range for growth is 23 to 43°C, and the pH range for growth is 6.0 to 9.0. These characteristics place it in group IV of the genus Pseudomonas as a subspecies of Pseudomonas diminuta. Strain BL072 shows many characteristics similar to Pseudomonas SY-77-1 (25), a patented strain (21) with glutaryl-acyltransferase activity, but BL072 differs in having lower β-lactamase activity and different growth characteristics on various nutrient media. BL072 is similar to Pseudomonas strain N176 reported by Aramori et al. (3). However, strain BL072 can grow at higher temperatures than can N176.

Cultivation of BL072. The medium on which strain BL072 grew most rapidly (maximum growth rate = 0.35 at 32 to 40°C [Fig. 2]) is a Luria-Bertani medium without added NaCl (seed medium). However, little glutaryl acylase activity was expressed under these conditions. A medium that was optimal for production of GI-ACA acylase activity (production medium) contained a low-salt casein hydrolysate as a key ingredient. Although the growth rate was maximal at 32 to 40°C, the maximal expression of acylase activity was found at 28°C (Fig. 2). Unlike the patented strain SY-77-1, which produced acylase optimally at pH 10 (20), strain BL072 produced acylase best between pH 7 and 8. This activity was not induced by 0.1% glutaric acid (Fig. 3). Acylase activity was not found in the supernatant of actively growing cells, but stationary-phase cells tended to lyse and release acylase activity.

The profile of a typical stirred-tank fermentation of BL072 is shown in Fig. 4. By comparing the development of acylase activity to the CO2 production and cell mass curves, acylase activity appeared to be growth associated. Acylase titer was nearly maximal (3 IU/liter) at the peak of CO2 production at 33 h. A rise in medium pH was seen during the growth phase, and if this was not controlled at pH 8.0 with sulfuric acid, the pH would rise to about 8.7, resulting in some loss of activity. The cell mass in this fermentation was low, at 5.5 g of cell dry weight per liter. The in-cycle feeding of this fermentation with both casein and glycerol resulted in a doubling of the cell mass but also caused a lowering of volumetric acylase activity (data not shown).

Isolation of mutants with increased acylase production. The parent strain BL072 was mutated with N-methyl-N'-nitro-N-nitrosoguanidine as described in Materials and Methods, and mutants were screened for increased acylase activity. A superior mutant was selected, and the mutation procedure was repeated on the superior strain for a total of seven times.

FIG. 2. Temperature dependence of growth rate and acylase production for BL072 parent and mutant strains. The specific growth rate (μ) in reciprocal hours is given for the original isolate (○) cultured in seed medium. The temperature dependence of acylase production for both the original isolate (●) and the mutant producing higher amounts of acylase (■) are normalized to the maximal activity of each strain. Maximal activities were 4.5 IU/liter for the original isolate and 22 IU/liter for the mutant.

FIG. 3. Effect of medium pH on acylase production. Acylase activity of the BL072 parent strain grown in production medium (○) or in production medium plus glutaric acid (1 g/liter) (●) with the stated pH before sterilization. See Materials and Methods for growth and assay conditions. Final medium pH after growth was 1.0 to 1.5 pH units higher for the cultures at pH 8.5 or below. Cultures did not grow well at pH 9.0 or above, and the medium pH did not change significantly for these samples.
FIG. 4. Fermentation profile of BL072 parent strain. Fermentation conditions and assay procedures are described in Materials and Methods. Carbon dioxide concentration is measured as a percentage of exhaust gas. Symbols: ○, cell dry weight; ●, IU/ml.

The temperature sensitivity of acylase production remained similar for the mutant producing high levels of acylase (Fig. 2). The fermentation profile of a hyperproducing mutant is shown in Fig. 5. Overall, the fermentations were similar, but the amount of acylase produced by the mutant was fourfold that produced by the original parent, even though the cell mass was increased by only 15% on a volumetric basis. This represents a 3.5-fold increase in specific activity with respect

FIG. 5. Fermentation profile of a hyperproducing mutant. Procedures and symbols are the same as in Fig. 3.
to cell mass. This mutation producing high acylase activity was stable through six serial subcultures.

Conversion of substrates by cells. Cells of BL072 were used to convert GI-ACA and related compounds to the β-lactam nucleus (e.g., 7-ACA and 7-ADCA). Although no activity was detected against cephalosporin C, GI-ADCA and GI-desacetyl-ACA were slightly better substrates for BL072 acylase than was GI-ACA (Table 1). Some of the product formed from GI-ACA was measured as desacetyl 7-ACA because of the presence of cell-associated esterase activity. The susceptible ester moiety is not present in the desacetyl and desacetoxy derivatives. This was an advantage when these latter compounds were used in screening programs, as esterase activity will not interfere with the product formation. An additional advantage to using the desacetyl and desacetoxy derivatives in screening is the increased chemical stability of these derivatives. Table 1 shows that GI-desacetyl-ACA and GI-ADCA are 5-fold and 24-fold more stable, respectively, than GI-ACA.

Additional proof of the authenticity of the GI-ACA acylase activity was that the three products formed above (7-ACA, 7-ADCA, and desacetyl 7-ACA) were all susceptible to hydrolysis by a cephalosporinase (Miles Laboratories #31-110-1).

It was noted that sonication and freeze-thaw treatments did not increase the expressed acylase activities of intact cell suspensions, suggesting that the acylase was located in the periplasm or another location accessible to GI-ACA.

Additional studies are being performed with this culture. Pilot plant-scale fermentations have been run with BL072 mutants, and the cell mass has been used as a source of GI-ACA acylase. A subsequent paper will deal with the purification and characterization of this acylase as well as its immobilization and use in converting GI-ACA in a column reactor. It is hoped that this acylase will be a step in the development of an economic process converting cephalosporin C to 7-ACA.

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