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Molecular Cloning and Analysis of the Gene Encoding the Thermostable Penicillin G Acylase from *Alcaligenes faecalis*

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***Alcaligenes faecalis* penicillin G acylase is more stable than the *Escherichia coli* enzyme. The activity of the *A. faecalis* enzyme was not affected by incubation at 50°C for 20 min, whereas more than 50% of the *E. coli* enzyme was irreversibly inactivated by the same treatment. To study the molecular basis of this higher stability, the *A. faecalis* enzyme was isolated and its gene was cloned and sequenced. The gene encodes a polypeptide that is characteristic of periplasmic penicillin G acylase (signal peptide-α subunit-spacer-β subunit). Purification, N-terminal amino acid analysis, and molecular mass determination of the penicillin G acylase showed that the α and β subunits have molecular masses of 23.0 and 62.7 kDa, respectively. The length of the spacer is 37 amino acids. Amino acid sequence alignment demonstrated significant homology with the penicillin G acylase from *E. coli*. A unique feature of the *A. faecalis* enzyme is the presence of two cysteines that form a disulfide bridge. The stability of the *A. faecalis* penicillin G acylase, but not that of the *E. coli* enzyme, which has no cysteines, was decreased by a reductant. Thus, the improved thermostability is attributed to the presence of the disulfide bridge.**

Penicillin G acylase (penicillin amidase or penicillin amidohydrolase; EC 3.5.1.11) is commercially used to hydrolyze benzylpenicillin to phenylacetic acid and 6-aminopenicillanic acid (6-APA), which is a key intermediate in the synthesis of a large variety of penicillins (12). The diverse group of penicillin acylases has been classified according to the substrates they prefer (17, 40). Class II penicillin acylases prefer penicillin G, while penicillin V and ampicillin are the substrates of choice for class I and III enzymes, respectively. Class II penicillin acylases can be subdivided into two groups: those hydrolyzing aromatic or phenylacetyl amides (IIa) and those hydrolyzing aliphatic amides (IIb). Class IIa enzymes that have been sequenced are those from the *Enterobacteriaceae* family members *Escherichia coli* (37), *Kluyvera citrophila* (3), and *Providencia rettgeri* (25) and from the gram-positive bacteria *Arthrobacter viscosus* (33) and *Bacillus megaterium* (28).

Besides having similar substrate preferences, these enzymes are evolutionarily related (3, 33). Their nucleotide sequences also suggest that all class IIa penicillin acylase genes encode a polypeptide that is subject to processing. The maturation of this polypeptide to the fully active enzyme has been studied in detail in *E. coli*. The polypeptide is transported to the periplasm (with concomitant removal of the signal sequence) and processed to a small α subunit (24 kDa) and a β subunit (63 kDa) by excision of a small spacer peptide (4, 5).

Recently, the three-dimensional (3D) structure of the *E. coli* penicillin G acylase was published (13). This structure supports the reaction mechanism proposed by Konecny (22), illustrates the role of some amino acid residues (27), and shows that the N-terminal serine is the only catalytic residue. The kinetics of the enzyme have been studied mainly with respect to the large-scale production of 6-APA. The K_m values reported for penicillin G hydrolysis by the *E. coli* enzyme vary from 7.7 mM to 4.6 μM (2, 6, 18, 26, 32, 39). The high K_m values are partly explained by product (phenylacetic acid) inhibition and by diffusion limitation effects (26). The K_m of the penicillin G acylase from *A. viscosus* is 0.42 mM (33), and that of the enzyme from *K. citrophila* is 0.02 mM (34). There is also considerable variation in the reported k_{cat} values. They range from 6.6 U · mg^{-1} (= 9.5 s^{-1}) to 59 U · mg^{-1} for the *E. coli* enzyme (26, 32). For the *A. viscosus* and *K. citrophila* penicillin acylases, values of 62 and 39 U · mg^{-1}, respectively, have been published.

A few studies on the thermostability of the enzyme have been performed. Erarslan and Koçer (14) have tried to stabilize the *E. coli* enzyme by glutaraldehyde cross-linking, but both wild-type and cross-linked penicillin G acylases showed 40 to 50% denaturation after 30 min of incubation at 45°C. After 30 min at 50°C, all activity was lost. Also, *A. viscosus* penicillin G acylase is rapidly inactivated at 50°C (33). The *K. citrophila* enzyme was reported to be more stable than its *E. coli* counterpart, but no experimental data were given to support these claims (1).

In this communication, we describe the purification of the *Alcaligenes faecalis* penicillin G acylase and the demonstration of its superior thermostability. The molecular cloning of its gene and determination of its structure and its amino acid composition led to an explanation for the higher stability of *A. faecalis* penicillin G acylase. We also report the kinetics of the protein with respect to the hydrolysis of penicillin G and 6-nitro-(3-phenyl-acetamido)-benzoic acid (NIPAB).

MATERIALS AND METHODS

Isolation and purification of *A. faecalis* penicillin G acylase. *A. faecalis* ATCC 19018 cells were grown for 24 h at 15 mg/ml of yeast extract buffered with phosphate and harvested by centrifugation. Cells were resuspended in 100 mM Tris-HCl buffer (pH 8.0) containing 0.2 mM EDTA and 20 μg of lysozyme per ml. After 2 h at 30°C, the cell debris was removed by centrifugation. The enzyme

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was purified to homogeneity by two chromatography steps. First, the supernatant was loaded on a carboxymethyl cellulose column in 50 mM Na acetate (pH 4.5) and eluted with the same buffer at 500 mM. Penicillin acylase active fractions were pooled and dialyzed. Subsequently, these fractions were loaded on hydroxyapatite gel (Biogel HTP; Bio-Rad) and eluted with a linear gradient of 0 to 300 mM sodium phosphate buffer (pH 7.0) (3). The *E. coli* enzyme was purified in accordance with published methods (24). Protein purity was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined by the Bradford method.

N-terminal sequence and mass determinations. The N termini of both subunits were determined as follows. Purified protein was loaded on an SDS-PAGE gel. After electrophoresis, the protein bands were transferred to a polyvinylidene difluoride membrane (Bio-Rad) by electroblotting. Bands representing the α and β subunits were cut out. The amino acid sequence of the N terminus was determined by automated Edman degradation with an Applied Biosystems 477A Sequenator. The masses of individual subunits were determined by electrospray mass spectrometry (Fisons) after high-pressure liquid chromatography purification.

Cloning and sequencing of the penicillin G acylase gene from *A. faecalis*. Chromosomal DNA of *A. faecalis* (ATCC19018) was isolated and partially digested with *Sau*3A. Fractions ranging from 4 to 7 kb were purified and ligated in *Bam*HI-opened vector pACYC184 (8). *E. coli* HB101 (*proA leuB thi recA*) cells transformed with these constructs were plated on medium containing phenylacetyl L-leucine (PAL) (15). Clones growing on the plates harbor the penicillin G acylase gene, which allows them to liberate the essential leucine. The gene was probed with oligonucleotide AF1 (5′-AGC AAC CTG TGG AGC A/C C/G C TGC CCG GAG TGC GT-3′). This oligonucleotide was derived from the N-terminal sequence of the β subunit of the *A. faecalis* penicillin G acylase by taking into account the high GC content of *A. faecalis*, the sequence of *E. coli* (substitution of Leu for Met can occur by only one base substitution), the uncertainty of the sixth residue (T or R), and the indication that the unknown residues were cysteines. One of the clones harbored the active penicillin G acylase activity on a 6.4-kb insert. A truncated plasmid was obtained by *Nde*I and *Sal*I digestion, filling in with DNA polymerase, and religation. This plasmid, essentially containing a 3.9-kb *Sau*3A-*Nde*I digest, also yielded enzyme activity, indicating that the complete gene is present. This 3.9-kb fragment was sequenced in pTZ18R and pTZ19R (Pharmacia) by using the dideoxy method.

Sequence analysis. DNA and protein sequences were analyzed by using the Wisconsin Genetics Computer Group (GCG) sequence analysis package, version 8.1. The DNA sequence identities of the complete coding sequence (signal-α subunit-spacer-β subunit) and the separate subunits were determined by using the algorithm of Needleman and Wunsch (31).

For the accession numbers of the penicillin G acylase sequences used for comparison, see Table 1. Please note that we used the GenBank entry of the *B. megaterium* gene which, in contrast to an earlier report (19), codes for a heterodimer (20).

Amino acid similarity and identity percentages were derived for the separate subunits by using the method of Smith and Waterman, which is available in the GCG program. In this algorithm, the similarity between amino acids (e.g., valine and alanine) is quantified on the basis of the evolutionary distance between those residues (16). The amino acid compositions of the proteins and their subunits were derived from the GenBank DNA files and the data reported in the accompanying papers.

The 3D structure of the *E. coli* penicillin G acylase, now available in the Brookhaven Protein Data Bank (1PNK, 1PNL, and 1PNM), was used to compare differences and similarities between the *A. faecalis* and *E. coli* enzymes (13).

Enzyme kinetics and thermostability. Enzyme activity was determined essentially as reported in the literature, by using NIPAB (Sigma, St. Louis, Mo.) in 50 mM phosphate buffer (pH 7.5) (24). The reaction volume was decreased to 1.0 ml. The penicillin G kinetics were determined in the same buffer. Samples of the reaction mixture were diluted 1:1 with 30% acetonitrile in 0.1 M KH_2PO_4 (pH 3)–0.075% SDS and loaded onto a CP-Microspher C_{18} column. The column was equilibrated and eluted (1 ml/min) with the same buffer. Detection was at 214 nm. The concentration was determined by comparison with standards with known concentrations. The thermostability of the *E. coli* and *A. faecalis* penicillin G acylases was determined as follows. A 20-μl sample of the enzyme dissolved in 100 mM sodium phosphate buffer (pH 7.5) was incubated for 0, 5, 10, or 20 min at 50 or 55°C. In the case of reduction of the enzyme, 10 mM dithiothreitol (DTT) was present in the phosphate buffer. After cooling of the samples, the activity was determined by using a fixed, saturating NIPAB concentration (0.2 mM). In these experiments, all activities were related to samples that were not subjected to temperature elevation. For determination of the V_{max} and K_m of the enzyme, the NIPAB concentration was varied between 0.2 μM and 1 mM. The kinetic parameters were obtained by using a Lineweaver-Burk plot. In all cases, hydrolysis of the substrate in the blanks was negligible.

Nucleotide sequence accession number. The nucleotide sequence of the *A. faecalis* penicillin G acylase gene has been assigned GenBank accession no. U93881.

RESULTS AND DISCUSSION

Purification and N-terminal sequencing of *A. faecalis* penicillin G acylase. Penicillin G acylase was purified from *A. faecalis* ATCC 19018. Typically, 1,000 U (23 mg) per liter was obtained. The purified enzyme showed two bands on SDS-PAGE: one of approximately 65 kDa and another of 25 kDa.

N-terminal sequence analysis showed that the small subunit was blocked. Most likely, the supposed N-terminal glutamine residue formed pyroglutamine, rendering the protein inaccessible to automated degradation. After treatment of the protein with pyroglutamate aminopeptidase, the sample was subjected to SDS-PAGE, electroblotted, and sequenced again. The N terminus, X_1QX_2VEVM (with X_1 equal to Gly, Ser, or Val), did not share significant similarity with sequences from the α subunits of penicillin acylases from other sources.

The N-terminal sequence of the large subunit was SNLWSTXPEXV. This sequence contains the important conserved N-terminal serine-asparagine doublet found in the β subunits of all of the penicillin G acylases sequenced so far. An oligonucleotide based on this sequence was synthesized as a probe to clone the penicillin G acylase gene from *A. faecalis*.

Isolation and DNA sequencing of the gene. The gene for *A. faecalis* penicillin G acylase was isolated by inserting partially digested chromosomal DNA into vector pACYC184. Transformants of *E. coli* HB101 were selected on PAL plates since leucine-deficient HB101 can grow only when enzyme-catalyzed PAL deacylation liberates the essential amino acid. Clone pAF1, which harbored a 6.4-kb insert, was selected for further analysis since it inhibited the growth of a *Serratia marcescens* strain which is resistant to penicillin G but sensitive to 6-APA (30). Oligonucleotide AF1, corresponding to the N-terminal end of the β subunit, was used to identify the gene. A 3.9-kb fragment of this insert comprising penicillin G acylase activity was subcloned and sequenced. The DNA sequence of the *A. faecalis* gene contains an open reading frame of 2,451 nucleotides. Comparison of the DNA identity levels of the penicillin G acylase genes with 16S rRNA sequences does not indicate unusual homology of the *A. faecalis* enzyme with any of the other sequences (Table 1). Thus, horizontal gene transfer between the members of the family *Enterobacteriaceae* is very unlikely. In contrast, the close relationship between the penicillin G acylases from *B. megaterium* and *A. viscosus* points to a horizontal gene transfer event (28).

Enzyme properties. Translation of the DNA sequence shows the typical polypeptide organization (signal sequence-α subunit-spacer-β subunit) observed for other penicillin G acylases. The total length of the coding sequence is 816 amino acids. The first stretch of 26 amino acids has the properties of a signal peptide (41), substantiating the periplasmic location of the enzyme. The predicted size of this signal sequence is in line with the determined N-terminal sequence of the α subunit. Determination of the size of the α subunit was not straightforward. SDS-PAGE indicates a molecular mass of approximately 25 kDa, but errors in molecular size determination made when using this technique have been reported (29). Therefore, the molecular mass of the α subunit was determined by using electrospray mass spectrometry. The spectrum showed an α-subunit mass of 22,982 Da. This value corresponds to a length of 202 amino acid residues (22,999 Da). Cyclization of the N-terminal glutamine to a pyroglutamate can account for the 17-Da decrease in molecular mass, since then the terminal amino group is removed. The length of the spacer between the C terminus of the α subunit and the N terminus of the β subunit is 37 amino acid molecules.

The size of the β subunit was derived from the DNA sequence by starting with the codon for the N-terminal serine and proceeding to the stop codon. Theoretically, the 551 amino acids result in a molecular mass of 62.7 kDa. Molecular mass determinations by SDS-PAGE and electrospray mass

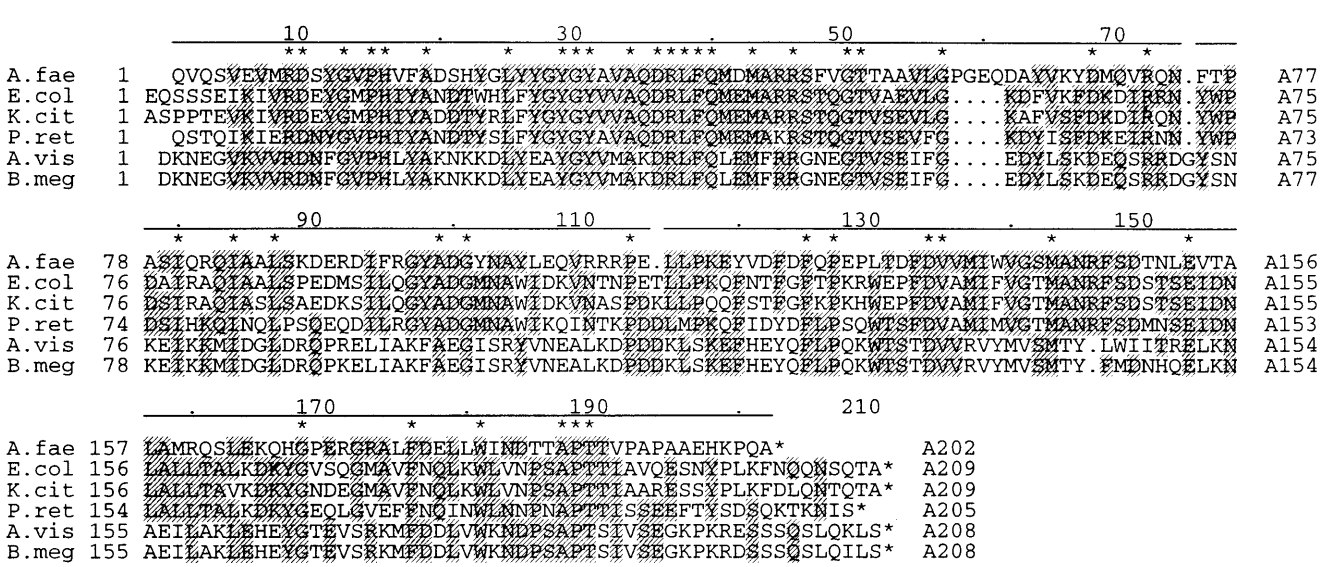

FIG. 1. Comparison of the amino acid sequence of *A. faecalis* penicillin G acylase with those of the penicillin G acylases of *E. coli*, *K. citrophila*, *P. rettgeri*, *A. viscosus*, and *B. megaterium*. Amino acids conserved in the enzymes of all six bacteria are marked by asterisks. Residues that are identical in at least three species are shaded, and when two different amino acids are each present in three enzymes, those present in *A. faecalis* are shaded. The two cysteines uniquely present in *A. faecalis* are circled.

spectrometry confirmed this size, with masses of 63 and 62.736 kDa, respectively. The sequences of both subunits are presented in Fig. 1.

The amino acid sequences of the α and β subunits were compared with those of the five other class II penicillin acylases that have been published (Table 1 and Fig. 1). Evidently, the amino acid sequence is more conserved than the DNA sequence. In both the α and β subunits, regions of conserved amino acids are observed. In the α subunit, these are residues A9 to A19 (i.e., residues 9 to 19 of the α subunit), A29 to A51, and A187 to A191. Surprisingly, amino acids A133 to A149 are highly conserved in the proteobacteria *A. faecalis*, *E. coli*, *K. citrophila*, and *P. rettgeri* but largely different in the gram-positive bacteria *A. viscosus* and *B. megaterium* (Fig. 1). Most of these amino acids, of which only aspartate (A134), valine (A135), and the well-studied methionine residue (A143) are present in all of the species, are located within 10 Å of the active-site serine. Within the group of proteobacteria, the insertion of four amino acids (A58 to A61) in the *A. faecalis* protein, compared to, e.g., *E. coli*, is striking. The residues flanking this insert are part of a turn between two α helices which can easily accommodate extra residues (13).

Homologous sequences can also be discerned in the β subunit. Most important is the N-terminal SN sequence, but other

TABLE 1. Properties and relationships of some class IIa penicillin G acylases[a]

Parameter	*A. faecalis*	*E. coli*	*K. citrophila*	*P. rettgeri*	*A. viscosus*	*B. megaterium*
% DNA identity of coding sequence	100	49	52	48	40	39
Molecular mass (kDa) of mature protein	85.7	86.2	85.2	86.1	85.7	85.6
No. of signal sequence aa[b]	26	26	26	23	26	26
α subunit						
% DNA identity	100	53	56	51	42	43
Molecular mass (kDa)	23.0	23.8	23.6	23.7	24.3	24.2
No. of aa	202	209	209	205	208	208
% aa identity	100	47	47	47	32	32
% aa similarity	100	67	65	68	58	58
No. of spacer amino acids	37	54	54	56	31	31
β subunit						
% DNA identity	100	49	52	48	41	39
Molecular mass (kDa)	62.7	63.4	61.7	62.2	61.4	61.4
No. of aa	551	557	555	553	537	537
% aa identity	100	41	41	40	34	33
% aa similarity	100	59	59	58	56	56

[a] The references and GenBank accession numbers, respectively, for the sequences compared are as follows: *A. faecalis*, this report and U93881; *E. coli*, 36 and X04114; *K. citrophila*, 3 and M15418; *P. rettgeri*, 21 and M86533; *A. viscosus*, 23 and L04471; and *B. megaterium*, 9 and U07682.

[b] aa, amino acids.

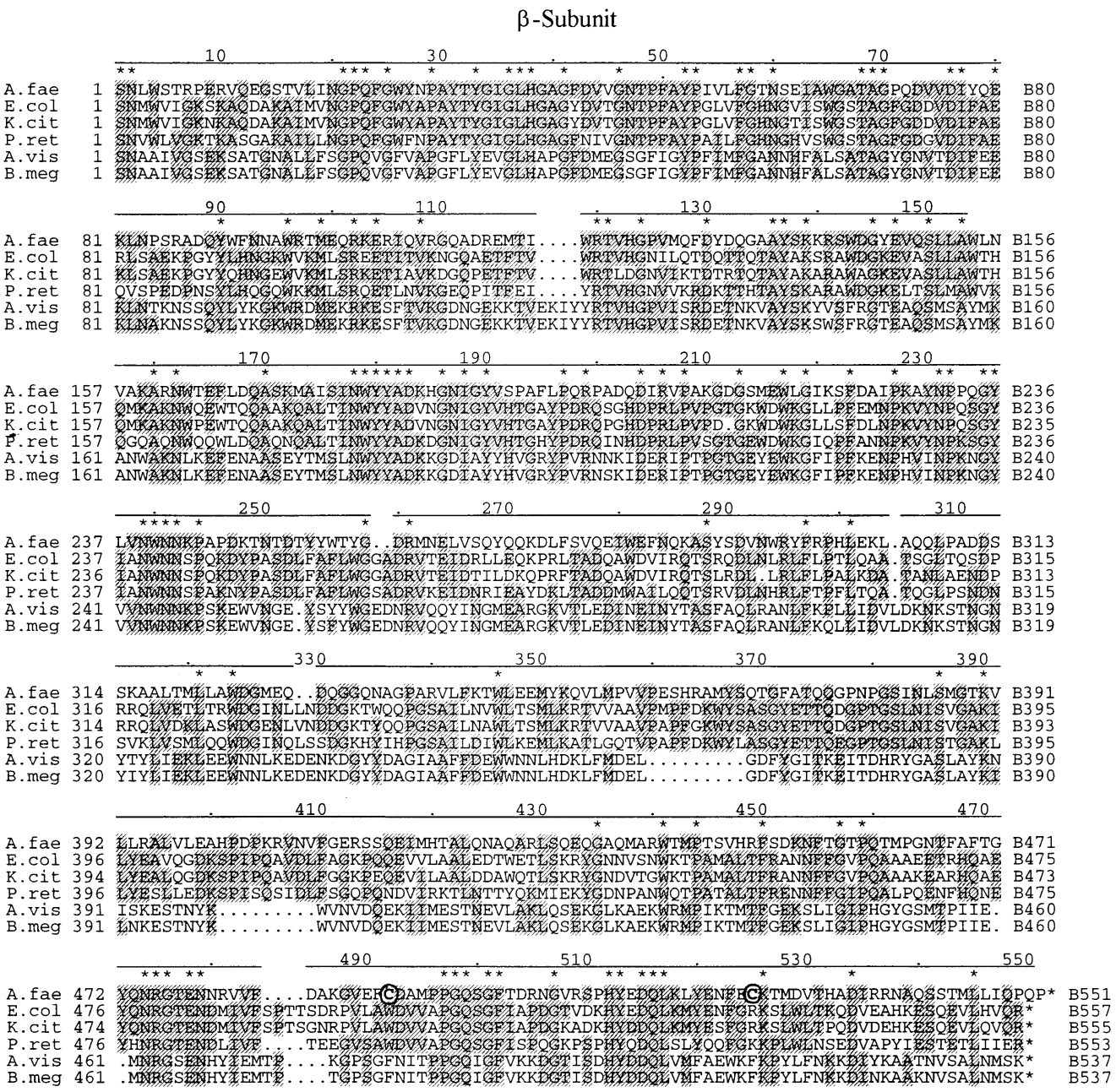

FIG. 1—*Continued.*

fully conserved regions are present in the first 75 amino acids of the β subunit, several of which participate in the active site. Other conserved clusters are B178 to B183, B227 to B244, B474 to B479, and B497 to B502. Except for the last cluster, all of these sequences are in the vicinity of the active site.

Some variation also exists in the amino acid number of the β subunit from our penicillin G acylase and that from *E. coli*. *A. faecalis* penicillin G acylase has a two-amino-acid deletion at residues B259 and B260. In the 3D structure, such a deletion can be compensated for by only a slight displacement of glycine B259. Another deletion of two amino acids, between residues B329 and B330, and one of four residues, almost at the C terminus (between B484 and B485; Fig. 1), are located at the surface of the protein. For these changes also, only small modifications in the *E. coli* structure are sufficient.

These considerations indicate that the 3D structure of the penicillin G acylase from *E. coli* is very useful to predict the structure of the *A. faecalis* enzyme, allowing us to compare the differences between the enzymes in more detail.

Enzyme activity. The question of whether the protein sequence differences affect the kinetic behavior of the enzyme arises. The V_{max} and K_m of the *A. faecalis* enzyme were determined by varying the NIPAB concentration between 0.2 μM and 1 mM and the concentration of penicillin G between 2.5 and 40 μM. The kinetic parameters were determined by using a Lineweaver-Burk plot of six different experiments.

TABLE 2. Kinetic parameters of some penicillin G acylases

Bacterium	NIPAB			Penicillin G		
	V_{max} ($U \cdot mg^{-1}$)	Turnover (s^{-1})	K_m (μM)	V_{max} ($U \cdot mg^{-1}$)	Turnover (s^{-1})	K_m (μM)
A. faecalis	35 ± 0.4	50 ± 0.7	5.1 ± 0.2	44 ± 2	63 ± 3	2 ± 0.2
E. coli[a]		25	15		48	4.6
K. citrophila[b]		27	11	40	56.5	15–20
P. rettgeri[c]				25		
A. viscosus[d]				62		420
B. megaterium[e]					51	4,500

[a] The penicillin values for *E. coli* were taken from Margolin et al. (26), and the NIPAB values are from Kutzbach and Rauenbusch (24).
[b] Taken from Prieto et al. (34), Roa et al. (36), and Barbero et al. (3).
[c] Calculated from the specific activity of a crude extract (compared to an *E. coli* extract) reported by Daumy and coworkers (11).
[d] Taken from Ohashi et al. (33).
[e] The V_{max} of the *B. megaterium* enzyme was calculated from the specific activity reported by Chiang and Bennett (9).

Whereas the K_m values for penicillin G hydrolysis by the *A. faecalis*, *E. coli*, and *K. citrophila* enzymes are of the same order of magnitude, the K_m values for *A. viscosus* and *B. megaterium* are considerably higher (Table 2). This similarity between the kinetic values of the proteobacterial enzymes is not surprising in view of the fact that of 32 residues within 10 Å of the active-site serine, only 3 differ. Interestingly, one of the three variable residues (B71) is important for substrate specificity. Experimental substitution (F71V) changes the *K. citrophila* enzyme from a class IIb (preferring penicillin G) to a class IIa (preferring aliphatic side chains) enzyme (36).

The *A. faecalis* penicillin G acylase probably has a disulfide bridge. In the *Alcaligenes* enzyme, residues 492 and 525 of the β subunit are cysteines (circled in Fig. 1). The question is whether they can form a disulfide bridge. We can estimate the distance between these residues based on the structure of the *E. coli* enzyme by using the coordinates of that protein (13). The cysteines of *A. faecalis* align with Trp500 and Arg533 in *E. coli* (Fig. 1). In the 3D structure of the *E. coli* enzyme, the Cβ atoms of these residues are only 3.8 Å apart. This distance allows the formation of a disulfide bond when both residues are replaced with cysteines. The *A. faecalis* enzyme was loaded onto an SDS-PAGE gel either in the presence (lane 1) or in the absence (lane 2) of the reductant DTT (Fig. 2). The electrophoretic mobility of the small α subunit was not affected by the presence of the reductant. However, the β subunit of the reduced sample showed a small but distinct and reproducible decrease in mobility. A change in electrophoretic mobility on SDS-PAGE due to reduction of the protein can be considered indicative of the presence of a disulfide bridge. In a similar experiment with the *E. coli* enzyme, no difference was observed between the reduced (lane 3) and oxidized (lane 4) samples. Thus, the mobility of the 63-kDa β subunit of the *A. faecalis* enzyme depends on its oxidation state whereas the mobility of the *E. coli* enzyme is not affected. Indeed, this indicates that the *A. faecalis* enzyme contains a disulfide bridge.

Increased thermostability of *A. penicillin* G acylase. Erarslan and Koçer (14) have attempted to increase the thermostability of the *E. coli* penicillin G acylase by cross-linking the enzyme with glutaraldehyde. The disulfide bridge naturally occurring in the *A. faecalis* enzyme may result in improved stability compared to other penicillin G acylases without the necessity for any chemical modification. Purified enzymes from both *E. coli* and *A. faecalis* were incubated at 50 and 55°C for 5 to 20 min, and the residual activity was measured at 37°C (Fig. 2). The activity of the *A. faecalis* enzyme after 5, 10, or 20 min of incubation at 50°C was hardly affected, whereas more than 50% of the *E. coli* protein became inactive after 10 min. The difference in stability at 55°C confirmed this observation. At that temperature, 50% of the *E. coli* activity was lost within 5 min. The half-life of the *A. faecalis* enzyme at 55°C was almost 15 min. Thus, the stability of the *A. faecalis* enzyme is significantly higher than that of the *E. coli* enzyme. Most likely, the disulfide bridge is responsible for this phenomenon. To substantiate this hypothesis further, we repeated the stability experiment in the presence of 10 mM DTT, which is known to reduce disulfide bridges. The stability pattern of the *E. coli* penicillin G acylase, which does not contain any disulfide bridges, was not altered by the reductant (Fig. 3). In contrast, the stability of the *A. faecalis* enzyme was seriously affected. The enzyme was stable at 50°C without DTT, but lost approximately 50% of its activity within 20 min when the reductant was present.

Concluding remarks. We have isolated and cloned the *A. faecalis* penicillin G acylase. At the protein level, the enzyme shows the highest degree of homology with the enzymes of the other gram-negative bacteria and the data show conserved regions dispersed over both subunits.

Schumacher and coworkers (37) showed that maturation of the penicillin G acylase in *E. coli* occurs in three steps: (i) removal of the signal sequence during transport to the periplasm, (ii) cleavage between the N-terminal serine of the β subunit and the spacer peptide, and (iii) removal of the spacer by cleavage at the C-terminal side of the α subunit. Recently, it was proposed that these proteolytic reactions are driven autocatalytically (7, 38). We have no evidence that the maturation process in *A. faecalis* is the same as in *E. coli*, but three observations support this assumption: (i) the similarity in polypeptide composition (signal peptide-α subunit-spacer-β subunit); (ii) the presence of glycine at position B21, which is necessary for maturation (35); and (iii) the possibility of heterologous *A. faecalis* penicillin G acylase expression in *E. coli.*

Despite its many similarities with other penicillin G acylases, the *A. faecalis* enzyme has a clear industrial advantage over other well-characterized penicillin acylases in β-lactam conversions because of its higher thermostability. This makes the *Alcaligenes* enzyme a more attractive biocatalyst both in hydrolysis and in synthetic conversions. The presence of two nonconserved cysteine residues at positions B492 and B525 in the

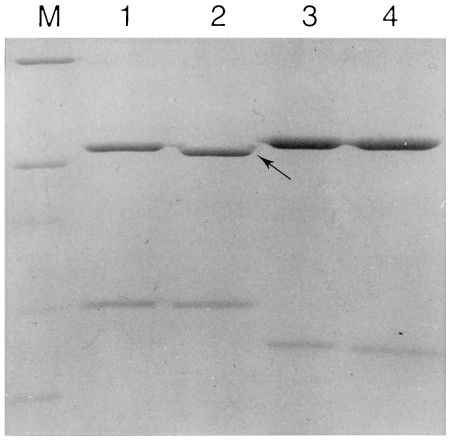

FIG. 2. SDS-PAGE of *A. faecalis* and *E. coli* penicillin G acylases. Lanes: 1, *A. faecalis* enzyme reduced; 2, *A. faecalis* enzyme oxidized; 3, *E. coli* enzyme reduced; 4, *E. coli* enzyme oxidized. The molecular masses of the marker proteins (lane M) are (from the top) 116.4, 55.6, 39.2, 26.6, and 20.1 kDa. The arrow points at the oxidized β subunit.

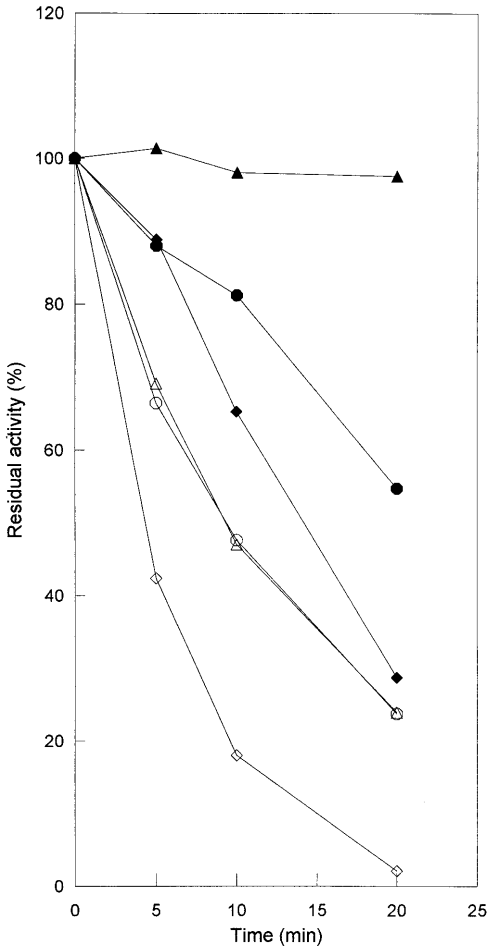

FIG. 3. Thermal stability of *A. faecalis* and *E. coli* penicillin G acylases under oxidative and reducing conditions. Samples were incubated at 50°C (▲, △) or 55°C (◆, ◇) to compare stability under oxidative conditions. The effect of reducing conditions is shown by the absence (▲, △) or presence (●, ○) of a reductant (10 mM DTT). Closed symbols represent the *A. faecalis* enzyme; open symbols represent penicillin G acylase from *E. coli*.

Alcaligenes enzyme probably accounts for this higher stability on the basis of the following observations: (i) a comparison with the known 3D structure of the *E. coli* enzyme indicates that these two cysteine residues are in close proximity, (ii) the β subunit of the *A. faecalis* enzyme shows an electrophoretic mobility that depends on its oxidation state, (iii) the *A. faecalis* enzyme is significantly more thermostable in its oxidized than in its reduced state, and (iv) the *A. faecalis* penicillin G acylase is more stable than the *E. coli* enzyme, which has no disulfide bridge. These four arguments led us to conclude that a disulfide bond accounts for the higher stability.

The DNA sequences reported for other penicillin G acylases do not translate to any cysteine residues at all, excluding any disulfide bonds in those enzymes. However, Daumy et al. (10) have reported the presence of a disulfide bridge in a penicillin G acylase generated in an evolution experiment in which *P. rettgeri* cells were grown on 6-bromohexamide. Unfortunately, only the DNA sequence of penicillin G acylase, evolved from the same strain but grown on a different substrate, has been published. This DNA sequence does not code for any cysteines.

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