In our previous report we described the unusual properties of an endopeptidase termed LPXTGase purified from *Streptococcus pyogenes* (1). This membrane-associated enzyme cleaves a common sequence motif, LPXTG, found near the C termini of precursor proteins programmed to be displayed on the cell surface and linked to the cell wall peptidoglycan. We discovered that the LPXTGase is highly glycosylated and contains hydrophobic amino acids with unusually high masses, which are not among the 20 common amino acids present in ribosomally translated proteins. The presence of unusual amino acids indicated the possibility that the core protein of the enzyme might be constructed entirely or in part through the well characterized nonribosomal peptide synthesis pathway, where amino acids are assembled into a peptide on amino acid-activating multienzyme templates. Examples are the syntheses of peptide antibiotics in Bacilli sp. (2–4), *Streptomyces* sp. (5–7), and *Amycolatopsis* sp. (8, 9), synthesis of a phytoionophore in *Tolypocladium niveum* (10), and synthesis of the immunosuppressant cyclosporin A in the fungus *Tolypocladium niveum* (11). Unusual amino acids could also be incorporated into the ribosomally translated core protein through post-translational modifications, however. Another unusual feature of the core protein of LPXTGase is that only seven different amino acids represent 93% of the 61 common residues present in the core protein. It is particularly striking that alanine accounts for nearly 40% of the common amino acids found in the protein. Over-representation of one or two amino acids within a peptide is a characteristic feature of nonribosomally constructed peptide antibiotics (4, 5). As there exists an intriguing possibility that the core protein is indeed constructed at least in part by a nonribosomal peptide synthesis pathway, we considered whether some of the alanine residues might be in the D-form, a hallmark feature of nonribosomal synthesis of peptide antibiotics (11, 12). This prompted us to search for the presence of D-alanine in the core protein of LPXTGase. In this report, we present strong evidence that alanine in the D-isomorph is present in this bacterial enzyme.

**EXPERIMENTAL PROCEDURES**

*Growth and [14C]Alanine Labeling of Cells—* S. pyogenes, strain D-471, was grown in 1 liter of Todd-Hewitt medium supplemented with 1% yeast extract. Cells were harvested by centrifugation at 8000 rpm when the absorbance of the culture at 600 nm reached 0.6. The cell pellets were suspended in 1 liter of a synthetic culture medium, and after adding 100 μCi of [14C]alanine (Sigma, catalog no. A-7428) to the culture, cells were grown for 1 h at 37 °C in a shaker-incubator. Cells were harvested by centrifugation, and the spent medium was saved. A freshly harvested batch of late exponential cells from 1 liter of culture was suspended in 400 ml of 30 mM Mes1 buffer, pH 6.3, containing 100 mM NaCl, and cells were collected by centrifugation. The composition (in mg/liter) of the synthetic medium is as follows: salts CaCl2·H2O (300), MgSO4·7H2O (200), KCl (200), NaCl (12,000), NaH2PO4 (120), Na2HPO4 (140), and FeSO4·7H2O (1); amino acids glutamine (2000), tryptophan (150), and all other amino acids (400) (alanine was omitted); sugars glucose (4000), ribose (200), and deoxyribose (200); bases adenine (20), guanine (20), cytosine (20), thymine (20), and uracil (20); vitamins biotin (0.05), folic acid (1), riboflavin (1), niacin (1), pantothenic acid (1), p-aminobenzoic acid (1), p-aminosalicylic acid (1), and thiamine (1). Tryptophan and bases were dissolved in 20 ml of 0.2 M HCl prior to mixing with other components. The final pH of the medium was adjusted to 7.4.

*Purification of [14C]Alanine-labeled LPXTGase—* About 3 g of the harvested cell pellets were suspended in 120 ml of 30 mM Mes buffer, pH 6.3. About 500 units of phage lysozyme from group C streptococcus (13) were added to the cell suspension, and the mixture was incubated with shaking at 37 °C for 90 min. The resulting cell lysate was centrifuged at 10,000 rpm for 20 min, and the supernatant (cytosol) was saved. The pellets containing cell ghosts were suspended in 60 ml of 20 mM Tris buffer, pH 7.6, containing 0.2% Brij 35, the suspension was sonicated for 40 s, and the sonicated suspension was stirred at 4 °C overnight. The suspension was centrifuged for 40 min at 10,000 rpm, and the supernatant (membrane extract) was collected. From the combined supernatants, [14C]Alanine-labeled LPXTGase was purified according to the procedure described previously (14). Briefly, the combined supernatants were applied to a DEAE-cellulose column equilibrated with 20 mM Tris buffer, pH 6.8, and the column was eluted with 20 mM Tris buffer, pH 7.6, containing 0.1% Brij 35. Fall-through eluant was concentrated by ultrafiltration using a YM-3 membrane, and the concentrated fall-through fraction was applied to a Sephadex G-50 column (49 × 2.5 cm) equilibrated with 20 mM Tris buffer, pH 7.6, containing 0.1% Brij 35. The column was then eluted with the same buffer. The gel filtration step was repeated.
Acid Hydrolysis of [14C]Alanine-labeled LPXTGase and Purification of [14C]Alanine—The [14C]-labeled LPXTGase eluting from the second G-50 column was concentrated to 4 ml by ultrafiltration using a YM-3 membrane. The enzyme solution was lyophilized, and the dried enzyme was dissolved in 0.5 ml of water. Five volumes each of ethanol and ethyl acetate were added to the enzyme solution, and the mixture was kept overnight at −20 °C. Flocculated enzyme aggregates were collected by centrifugation. The solvents were removed from the enzyme pellets by means of Speedvac. The dried enzyme was dissolved in 300 μl of 80% phenol, after which the enzyme solution was mixed with 3 ml of 6 M HCl in capped glass vials. The enzyme was hydrolyzed by placing the vials in a 110 °C oven for 22 h. The acid hydrolysate of the enzyme was lyophilized to remove HCl and phenol. The dried enzyme hydrolysate was dissolved in 1 ml of 50% ethanol, and the hydrolysate was banded on a 20 × 20-cm silica gel plate. The plate was developed with a solvent mixture consisting of chloroform/methanol/water (1:2:1). An autoradiogram was made from the plate, and the [14C]-labeled alanine band was detected with a 20-cm silica gel plate (2:1:1). The peak enzyme fractions were concentrated and chromatographed again on the same G-50 column. From each fraction, a 50 μl aliquot was used for enzyme assay, and 200 μl was used for [14C] counts.

Detection of D-Alanine—In an initial experiment, 60 μl of an aliquot of [14C]-labeled alanine sample (18,200 cpm) was introduced into a microcentrifuge tube, then 4 μl of 1 M Tris buffer, pH 8.5, 0.2 unit of d-amino acid oxidase (EC 1.4.3.3, Sigma catalog no. A 5222) in 2 μl, and 0.2 unit of catalase (EC 1.11.1.6, Sigma catalog no. C 30) in 2 μl were added, and the reaction mixture was incubated for 2 h at room temperature. Authentic L-alanine with similar radioactivity was subjected to acid hydrolysis of the enzyme was lyophilized to remove HCl and phenol. The dried enzyme hydrolysate was dissolved in 1 ml of 50% ethanol, and the hydrolysate was banded on a 20 × 20-cm silica gel plate. The plate was developed with a solvent mixture consisting of chloroform/methanol/water (1:2:1). An autoradiogram was made from the plate, and the [14C]-labeled alanine band was located. The alanine band was scraped off the plate, silica gel was placed in a small column, and the column was eluted with 50% ethanol. The eluted material was dried by means of Speedvac, and the [14C]-labeled alanine was dissolved in 300 μl of distilled water.

RESULTS

14C Labeling of Cytosol and Membrane Extracts—To specifically label the alanines in the LPXTGase, we grew streptococci in the presence of [14C]alanine. Of the initial [14C]alanine in the synthetic medium totaling 1.118 × 10⁹ cpm, the cells incorporated 1.68 × 10⁷ cpm, or 15%, of the radioactive alanine. The combined radioactive counts in the cytosol and membrane
extract of these cells, which also contained free alanine, was 1.26 × 10^6 cpm.

**Purification of [14C]Alanine-labeled LPXTGase**—Fig. 1 shows the elution pattern of enzyme activity from the second Sephadex G-50 column. The enzyme activity profile, as detected by cleavage of a bead-bound 125I-labeled LPXTGase-containing peptide (13), coincided precisely with the 14C activity profile. The total radioactive counts of the purified LPXTGase were 1.42 × 10^5 cpm. Consistent with our previous findings that the LPXTGase does not contain any aromatic amino acid and thus does not absorb UV light at 280 nm, the enzyme peak showed no UV absorption at 280 nm, verifying the purity of the enzyme.

**Purification of [14C]Alanine from Acid Hydrolysate of LPXTGase**—Purified LPXTGase was hydrolyzed with 6 N HCl as described under “Experimental Procedures,” and the acid hydrolysate was subjected to silica gel thin layer chromatography using chloroform/methanol/water (1:2:1) as the running solvent. Fig. 2 shows that 14C radioactivity was nearly exclusively present in alanine (1.14 × 10⁵ cpm). A very faint radioactive band was seen at rf 0.75, which corresponded with glutamic acid. No other radioactive bands corresponding to the rest of the LPXTGase-constituent amino acids were detected. Mobilities of non-radioactive, LPXTGase-constituent amino acids on silica gel thin layer chromatography with the same solvent mixture were previously determined (results not shown).

**Presence of D-Alanine in the LPXTGase**—Treatment of d-alanine with d-amino acid oxidase coupled with catalase resulted in the deamination of D-alanine to produce pyruvic acid, a reaction that does not occur when L-alanine is similarly treated. In Fig. 3A, d-amino acid oxidase-catalase-treated [14C]alanine from the enzyme was loaded onto spot 1, similarly treated authentic L-[14C]alanine onto spot 2, untreated L-[14C]alanine onto spot 3, and authentic [14C]pyruvic acid was loaded onto spot 4 at the origins of a silica gel thin layer plate, and the plate was developed with chloroform/methanol/water (1:2:1). As shown, d-amino acid oxidase-catalase treatment of the [14C]alanine sample produced two radioactive products, a and b, of which the mobility of product a is identical with that of pyruvic acid. Product b is consistent with the mobility of the dimeric form of pyruvic acid. The yield of these products was low as reflected in the quantity loaded on the plate.

To verify the presence of the dimeric form of pyruvic acid, the remaining (80% of the total) [14C]alanine from the enzyme was incubated with an excess amount of d-amino acid oxidase and catalase for a prolonged time to ensure conversion of all D-alanine to pyruvic acid, and the reaction products were chromatographed on a silica gel plate in the same manner (Fig. 3B). The same products a and b were again detected but in higher yield. These two products were eluted from the plate and along with authentic pyruvic acid were applied to a silica gel plate, which was developed with ethyl acetate/propionic acid/water (2:1:1) (Fig. 3C). The main radioactive spots from both products a and b showed the same mobility as authentic pyruvic acid, but minor secondary radioactive spots were also observed. The fact that products a and b from Fig. 3B gave rise to nearly identical radioactive spots on subsequent chromatography us-
of the monomeric (a) and dimeric (b) forms of pyruvic acid. We show here that at least 27% of the alanine present in LPXTGase became deaminated by D-amino acid oxidase, verifying that LPXTGase does in fact contain D-amino acids. Based on radioactive count data, the estimated number of D-alanine residues in LPXTGase is 6–7. The possibility exists, however, that the actual number is higher because pyruvic acid decomposes at 165°C, and the hot air stream used during loading of the D-amino acid oxidase reaction products on the TLC plates may have decomposed some pyruvic acid.

All of the amino acids in proteins exhibit the same steric configuration; thus, they are all L-amino acids. D-Amino acids have not been identified in proteins, although they do exist in nature in peptides from multicellular organisms (16). In bacteria for instance, D-amino acids are commonly found in association with the amino acids used to construct the peptidoglycan. For example, in the disaccharide-pentapeptide precursor for peptidoglycan synthesis one D-glutamate and two D-alanines are present prior to cross-linking after which, the terminal D-alanine is removed to facilitate the linkage. However, beside the bacterial peptidoglycan, D-amino acids have generally been associated with non-ribosomal peptide synthesis, in the production of peptide antibiotics (2–9). Our finding of D-alanine in the LPXTGase enzyme still does not resolve whether the D-amino acid was incorporated through a non-ribosomal peptide synthesis pathway or was post-transcriptionally added to a ribosomally translated protein backbone. To date, the largest non-ribosomally synthesized peptide is <50 amino acids, and none have been found to exhibit catalytic activity. Because D-amino acids cannot be accommodated during ribosomal synthesis of a polypeptide chain, this strongly suggests that the 60-amino acid LPXTGase may be the first reported instance of a D-amino acid-containing enzyme that is not ribosomally synthesized.

One could argue, however, that the LPXTGase used in this paper might be contaminated by L-alanine oxidase or racemase. This is unlikely because the LPXTGase used in the amino acid analysis was highly purified as described previously (1). However, even if the LPXTGase were contaminated by a small amount of impurities, it is unlikely that L-alanine oxidase or racemase is a part of the impurity. The L-alanine racemase from Escherichia coli (and other species) contains 359 amino acid residues and 40 acidic amino acid residues. Thus, L-alanine oxidase or racemase cannot appear in the fall-through fractions in DEAE-cellulose chromatography or the M₉₀ = 14,000 region of the G 50 chromatography used in purifying the LPXTGase. Furthermore, if L-alanine oxidase or racemase copurified with LPXTGase for inexplicable reasons, only free amino acids, and not amino acids contained in proteins, are substrates for these enzymes.

Although the deaminated product of D-alanine is pyruvic acid, we did observe an additional product. The keto-enol tautomerism of α-keto acids is well known (17). For example, pyruvic acid as a keto acid is expected to undergo keto-enol tautomerism, of which the enol form is an α-hydroxy acid that, as a rule, undergoes dimerization and polymerization. [14C]pyruvic acid is generally shipped as a lyophilized powder in a sealed vial. When freshly dissolved in 50% ethanol and chromatographed, a single radioactive spot is usually observed (Fig. 3, A and B). However, after standing it may become identical to the migration pattern of derivatives a and b in track 1. Taken together, these results indicate that pyruvic acid exists both in monomer and dimer forms.

**DISCUSSION**

D-Amino acid oxidase (EC 1.4.3.3) deaminates D-amino acid to the corresponding α-keto acids, but the enzyme is not reactive with L-amino acids (15). We show here that at least 27% of the alanine present in LPXTGase became deaminated by D-amino acid oxidase, verifying that LPXTGase does in fact contain D-amino acids. Based on radioactive count data, the estimated number of D-alanine residues in LPXTGase is 6–7. The possibility exists, however, that the actual number is higher because pyruvic acid decomposes at 165°C, and the hot air stream used during loading of the D-amino acid oxidase reaction products on the TLC plates may have decomposed some pyruvic acid.

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oxidized, and additional spots will appear after chromatography (Fig. 3C). Thus, we interpret that the secondary product that we observed in our chromatograms arose from the dimerization of pyruvic acid. Whatever caused the appearance of the second spot from the d-amino acid oxidase-treated [14C]alanine from LPXTGase, an identical second spot also appeared from authentic [14C]pyruvic acid. Therefore, it seems safe to conclude that the second spot originated from pyruvic acid.

Thus, in summary, we have identified the presence of at least six D-alanine residues in the LPXTGase enzyme, accounting for \( \approx 10\% \) of the total amino acids in this molecule. However, our results are inconclusive as to whether the D-alanine found in LPXTGase is the result of a post-translational modification of L-alanine to D-alanine or is incorporated non-ribosomally.

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