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Purification and Characterization of EpiD, a Flavoprotein Involved in the Biosynthesis of the Lantibiotic Epidermin

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The plasmid-encoded epidermin biosynthesis gene, *epiD*, of *Staphylococcus epidermidis* Tü3298 was expressed in *Escherichia coli* by using both the *malE* fusion system and the T7 RNA polymerase-promoter system. EpiD was identified by Western blotting (immunoblotting) with anti-maltose-binding protein (MBP)-EpiD antiserum. EpiD and the MBP-EpiD fusion protein, which were mainly present in the soluble protein fraction, were purified from the respective *E. coli* clones. Purified EpiD showed the typical absorption spectrum of an oxidized flavoprotein with maxima at 274, 382, and 453 nm. The coenzyme released from EpiD by heat treatment was identified as flavin mononucleotide. *S. epidermidis* Tü3298/EMS11, containing a mutation within *epiD*, was unable to synthesize active epidermin. This mutated gene, *epiD, was cloned in *E. coli* and expressed as an MBP-EpiD* fusion protein. DNA sequencing of *epiD** identified a point mutation that led to replacement of Gly-93 with Asp. Unlike MBP-EpiD, the fusion protein MBP-EpiD* could not bind flavin mononucleotide. We propose that EpiD catalyzes the removal of two reducing equivalents from the cysteine residue of the C-terminal *meso*-lanthionine to form a —C=C— double bond and is therefore involved in formation of the unusual *S*-[(*Z*)-2-aminovinyl]-D-cysteine structure in epidermin.**

Various staphylococci produce small, highly modified peptides that exert antibiotic activity mainly against gram-positive bacteria. These lantibiotics, which have lanthionine as a characteristic component, are ribosomally synthesized as prepeptides and posttranslationally converted into the mature peptides. The structures and prepeptide sequences of the following lantibiotics from various staphylococcal strains have been determined (reviewed in reference 13): epidermin (1, 33), gallidermin (18, 32), and Pep5 (17, 19). Additional lantibiotics are produced by a variety of microorganisms, such as lactococci, bacilli, and actinomycetes (reviewed in reference 16). The cationic lantibiotics interact with cytoplasmic membranes of bacteria and form transient, potential-dependent pores, thus causing efflux of ions and small molecules (21, 27).

Epidermin, which is produced and secreted into the medium by *Staphylococcus epidermidis* Tü3298, can be purified by reversed-phase high-performance liquid chromatography (12). The optimization of fermentative production leads to a large increase in the epidermin yield (14). Epidermin consists of a polycyclic heterodet peptide possessing thioether amino acids, such as *meso*-lanthionine and 3-methyllanthionine, which form four intramolecular thioether bridges. Moreover, epidermin contains the α,β-unsaturated amino acid didehydroaminobutyric acid and *S*-[(*Z*)-2-aminovinyl]-D-cysteine (1). Epidermin is ribosomally synthesized, as shown by the cloning and sequencing of the structural gene, *epiA* (33). Recently, it was shown that *epiA* is the first gene of an operon comprising *epiA*, *epiB*, *epiC*, and *epiD*. Two additional genes, *epiQ* and *epiP*, are transcribed in the opposite direction (31). All genes are located on the 54-kb plasmid pTü32 of *S. epidermidis* Tü3298. The involvement of the gene products in epidermin biosynthesis was verified by complementation analysis of *S. epidermidis* mutants (3, 4) and by heterologous gene expression in *Staphylococcus carnosus* (31).

Epidermin is synthesized as a 52-amino-acid prepeptide (N-terminal leader peptide with amino acids −30 to −1 and C-terminal segment with amino acids +1 to +22), which is posttranslationally modified and processed to the mature tetracyclic 22-amino-acid peptide antibiotic (33). The proposed modifying reactions comprise dehydration of serine and threonine residues, sulfide bridge formation, and oxidative decarboxylation at the C terminus (very likely catalyzed by EpiB, EpiC, and EpiD). So far, lantibiotic synthesizing enzymes have not been purified, nor have experimental data been presented elucidating the roles of the enzymes in lantibiotic biosynthesis.

Here we report the cloning and overexpression of *epiD* in *Escherichia coli*, the raising of anti-EpiD polyclonal antibodies, the purification of EpiD and a maltose-binding protein (MBP)-EpiD fusion protein, and the coenzyme flavin mononucleotide (FMN) dependence of the enzymes. Furthermore, we identify the genetic basis of the *epiD* mutation of *S. epidermidis* Tü3298/EMS11 and its effect on the FMN binding of MBP-EpiD. Based on the conclusion that EpiD is a flavoprotein, we propose a model for its function in epidermin biosynthesis.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *epiC* and *epiD* genes from *S. epidermidis* Tü3298 (DSM 3095) were cloned in *E. coli* K38 (26) with the plasmids pT7-5 (34) and pGP1-2 (8) for gene expression studies. *S. epidermidis* Tü3298/EMS11 carries a mutated *epiD* (*epiD**). *E. coli* TB1 [*ara* Δ(*lac proAB*) *rpsL* (φ80 *lacZ*ΔM15) *hsdR*] (15) and the vector pIH902 (a derivative of the vector pIH821 [25]) were obtained from New England BioLabs (Schwalbach, Germany). *E. coli* and *S. epidermidis* were cultivated in B-broth (10 g of casein hydrolysate 140 [GIBCO, Eggenstein-Leopoldshafen, Ger-

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many], 5 g of yeast extract [Difco], 5 g of NaCl, 1 g of glucose, and 1 g of K_2HPO_4 per liter [pH 7.3]). Unless otherwise stated, the growth temperature was 37°C.

DNA preparation, transformation, and molecular biological techniques. Staphylococcal DNA was prepared by the cleared-lysate method (24). Cells were lysed by the addition of lysostaphin (8 μg/ml), and the DNA was isolated by CsCl centrifugation. *E. coli* supercoiled plasmid DNA was prepared by the modified alkaline lysis method (6). *E. coli* was transformed by electroporation (10). Established protocols were followed for molecular biological techniques (28). Enzymes for molecular cloning were obtained from Boehringer (Mannheim, Germany), BRL (Eggenstein, Germany), and Pharmacia LKB (Freiburg, Germany); assay conditions were as recommended by the suppliers.

DNA sequencing and amplification by the polymerase chain reaction (PCR). The *malE-epiD* and *malE-epiD** fusions were sequenced by double-stranded DNA sequencing (9) by using the dideoxy procedure (29), the Pharmacia AutoRead Sequencing kit, and the A.L.F. DNA Sequencer from Pharmacia LKB. The following sequencing primers were labeled with fluorescein amidite: (i) a *malE* primer, used for sequencing from the 3′ terminus of *malE* across the polylinker [5′ d(GGTCGTCAGACTGTCGATGAAGCC) 3′]; (ii) an *epiD-1* primer, used for sequencing from the 5′ region to the 3′ terminus of *epiD* [5′ d(C(5881)ACTACATAGTTGAGTT AAAGCAAC(5905)) 3′]; (iii) an *epiD-2* primer, used for sequencing from the middle of *epiD* into the 3′-pIH902 polylinker region [5′ d(G(6058)TATTACCTGCATCAGC AAATAC(6080)) 3′]; (iv) an IH-rev primer, used for sequencing from the pIH902 polylinker into the 3′ terminus of *epiD* in the *malE/epiD* fusion [5′ d(CAAGCTTGCCTGCAG GTCG) 3′]. The numbers within parentheses refer to published *epiD* nucleotide sequence (31).

epiD was amplified by the PCR as described previously (5) with Vent-DNA-polymerase, pT*epiABCDQ* (4) as a template, and the following primers: (i) a primer located at the *epiD* 5′ terminus [5′ d(ATGTATGGAAAATTATTGATAT GCGC) 3′, containing the ATG start codon of *epiD*], and (ii) a primer located at the *epiD* 3′ terminus [5′ d(GACCTTTGT TAATCCAAAGGTC) 3′; TTA represents the TAA stop codon of *epiD*]. DNA was synthesized with Gene Assembler Plus from Pharmacia. Primers were purified by 13 or 15% acrylamide gel electrophoresis; the primer concentration was determined by the A_{260}.

Expression studies using the T7 RNA polymerase-promoter system. The T7 expression system described by Tabor and Richardson (34) was used. *epiC* and *epiD* were cloned into the T7 promoter-containing vector pT7-5. The resulting recombinant plasmids (Fig. 1) were transformed into *E. coli* K38 containing plasmid pGP1-2. Expression was measured by labeling the induced gene products with [^{35}S]methionine (51.8 TBq/mmol, 0.37 GBq/ml, in vivo cell labeling grade; Amersham). For selective labeling of the T7 promoter-controlled gene products, *E. coli* RNA polymerase was inhibited by rifampin (200 μg/ml).

Construction and purification of the MBP-EpiD fusion protein. *epiD* was amplified by the PCR and inserted in the polylinker *Stu*I site of the MBP (encoded by *malE* of *E. coli*) vector pIH902. The polylinker is located between *malE* and *lacZα*; in front of the polylinker is an oligonucleotide encoding polyasparagine and a factor Xa site. These sequences allow the removal of the MBP portion of the fusion protein. Expression of the fusion proteins is controlled by the inducible *tac* promoter (2). The vector pIH902 has an exact deletion at the *malE* signal sequence, which leads to a cytoplasmic location of the fusion protein. The fusion protein was expressed and purified as described by Riggs (25). Plasmid pIH902-*epiD* was transformed into *E. coli* TB1; transformants were selected with ampicillin (100 μg/ml) and identified by using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and isopropyl-β-D-thiogalactopyranoside (IPTG) (80 μg/ml and 0.1 mM, respectively). White clones were picked and tested for expression of the MBP-EpiD fusion protein. A 1% inoculated culture grown to a density of 0.45 (A_{600}) was then induced by the addition of 0.3 mM IPTG and cultivated for another 2 h. A 300-μl sample of the cell culture was centrifuged, and the pellet was resuspended in 100 μl of Laemmli sample buffer (22) and boiled for 5 min; an aliquot was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting. *E. coli* TB1(pIH902) was used as a control.

For purification of the MBP-EpiD fusion protein, IPTG-induced cells were centrifuged, the pellet was resuspended in column buffer (10 mM phosphate, 0.5 M NaCl, 1 mM sodium azide, 10 mM 2-mercaptoethanol, 0.25% Tween 20), and the cells were disrupted with glass beads (0.22-mm diameter). After two centrifugation steps (15 min at 6,000 × *g* and 60 min at 150,000 × *g*), the cell extract was applied to amylose resin equilibrated with column buffer. Unbound proteins were removed with 5 volumes of column buffer, and the resin was then washed with 5 volumes of column buffer lacking Tween to remove the detergent. Amylose-bound proteins were eluted with 10 mM maltose in column buffer lacking Tween and monitored by A_{280} and A_{450}. The residual *malE* expression was largely repressed by the addition of glucose to the medium.

Immunization procedure. For primary immunization, New Zealand White rabbits were injected subcutaneously with 1 mg of MBP-EpiD fusion protein dissolved in 1.5 ml of phosphate-buffered saline and emulsified in Freud's complete adjuvant. After 4 weeks, the rabbits were given intramuscular booster injections of 100 μg of protein in phosphate-buffered saline; the antisera were collected after three booster injections at 1-week intervals.

Factor Xa cleavage. Purified MBP-EpiD fusion protein was dialyzed against factor Xa buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 2 mM $CaCl_2$), and the protein concentration was adjusted to 1 mg/ml. After factor Xa (1 U of factor Xa per 100 μg of fusion protein) was added, the mix was incubated for several hours at 25°C. Cleavage was monitored by SDS-PAGE and Western blotting.

Purification of EpiD from *E. coli*. *E. coli* K38(pGP1-2, pT7-5*epiD*) was aerobically cultivated in 2.5 liters at 30°C to an A_{600} of 0.45. For induction of T7 RNA polymerase, the temperature was shifted to 42°C for 30 min and cultivation was then continued at 37°C for 2 h. All subsequent steps were performed at 4°C. Cultures were harvested by centrifugation. Four weight equivalents of glass beads and 2 volumes of 20 mM Tris-HCl (pH 8.0) buffer were added to the cell pellet. The cells were disrupted in a Corex test tube by vortexing for 2 min. Glass beads and unbroken cells were pelleted by centrifugation at 6,000 × *g* for 15 min. The supernatant was centrifuged at 150,000 × *g* for 1 h, and the pelleted membrane fraction was discarded. The cytosolic fraction was subjected to DEAE-Sepharose Fast Flow chromatography (20 ml of wet column resin) in a column equilibrated with 20 mM Tris-HCl (pH 8.0). Proteins were eluted with a linear gradient of 0 to 500 mM NaCl in 10 bed volumes of 20 mM Tris-HCl (pH 8.0) with a flow rate of 120 ml/h. Elution of EpiD was followed at A_{280} and A_{450} (absorption maximum of FMN), and the fractions were analyzed by

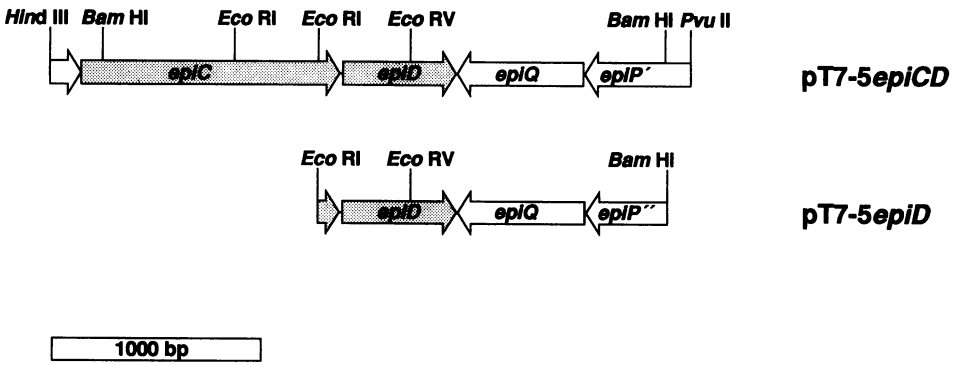

FIG. 1. DNA fragment of pTü32 showing the *epiC* and *epiD* region. The 3.1-kb *Hin*dIII-*Pvu*II fragment and the 1.7-kb *Eco*RI-*Bam*HI fragment were inserted in the expression vector pT7-5.

SDS-PAGE and Western blotting. Fractions with the highest EpiD content were pooled, diluted with 4 volumes of 20 mM Tris-HCl (pH 8.0), and loaded onto a Mono-Q column (HR 10/10; 10-ml bed volume). Proteins were eluted again with a linear gradient of 0 to 500 mM NaCl in 200 ml of 20 mM Tris-HCl (pH 8.0). The pooled EpiD-containing fractions were mixed with 1 volume of 1.7 M ammonium sulfate in 50 mM sodium phosphate buffer (pH 7.0) and then applied to Phenylsuperose HR 5/5 (1-ml bed volume), which was equilibrated with 0.85 M ammonium sulfate in 50 mM sodium phosphate buffer (pH 7.0). Proteins were eluted with 15 ml of a decreasing gradient of 0.85 to 0 M ammonium sulfate in 50 mM sodium phosphate buffer and then with constant 50 mM sodium phosphate buffer. Protein concentration was determined by the method of Bradford (7).

SDS-PAGE. Proteins were separated by tricine–SDS–10% PAGE (30) under reducing conditions. Prestained molecular weight markers from BRL were used as standards.

Western blotting. After SDS-PAGE, proteins were electrophoretically transferred to a Hybond-ECL nitrocellulose membrane (35). MBP fusion proteins were detected with anti-MBP antibodies (diluted 1:10,000) from New England BioLabs. EpiD was detected by anti-MBP-EpiD antiserum (diluted 1:2,500) raised in rabbits with purified MBP-EpiD fusion protein. Membranes were incubated with primary antibodies for 2 h and then incubated for 1 h with anti-rabbit immunoglobulin G-biotin conjugate (1:2,000) and 20 min with streptavidin-biotinylated horseradish peroxidase complex (1:3,000). Immunoreactive proteins were visualized by enhanced chemiluminescence with Hyperfilm-ECL (Amersham).

Protein sequence analysis. Automated Edman degradation was performed in a pulsed-liquid protein sequencer 477A (Applied Biosystems) equipped with an on-line phenylthiohydantoin amino acid analyzer 120A (Applied Biosystems). All reagents and solvents were from Applied Biosystems. An 8-μg sample of purified EpiD (400 pmol) dissolved in 30 μl of 50 mM phosphate buffer (pH 7.2) was applied to a glass fiber filter activated with trifluoroacetic acid; no Polybrene was applied. Sequencing was carried out without precycling with the standard programs BEGIN-1 and NORMAL-1.

Flavin identification. The coenzyme could be directly removed from purified EpiD by heat denaturation (8 min, 100°C). The MBP-EpiD fusion protein was precipitated by heat denaturation (8 min, 100°C), and the coenzyme was removed by treatment with cold 5% trichloroacetic acid and subsequent neutralization as described previously (23). The coenzyme was analyzed by thin-layer chromatography on cellulose-aluminum sheets with the following three solvents (20): (i) *n*-butyl alcohol–acetic acid–water (4:3:3), (ii) 5% Na_2HPO_4 in water, and (iii) *tert*-butyl alcohol–water (6:4). FMN, flavin adenine dinucleotide, and riboflavin were used as reference substances. All steps were carried out in the dark.

Chemicals and enzymes. Vent-DNA-polymerase, amylose resin for affinity chromatography, factor Xa, MBP used as a standard, and plasmid vector pIH902 were obtained from New England BioLabs. Tris, bovine serum albumin, FMN, and flavin adenine dinucleotide were obtained from Boehringer GmbH. The A.L.F. sequencing kit, fluorescein amidite (FluorePrime), DEAE-Sepharose Fast Flow, Mono-Q HR 10/10, and Phenylsuperose HR 5/5 were obtained from Pharmacia. IPTG, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, ampicillin, tricine, anti-rabbit immunoglobulin G-biotin conjugate, amino acids, and riboflavin were obtained from Sigma (Deisenhofen, Germany). [^{35}S]methionine, streptavidin-biotinylated horseradish peroxidase complex, the enhanced chemiluminescence Western blotting detection system, and Hybond-ECL nitrocellulose were obtained from Amersham (Braunschweig, Germany). Prestained protein molecular mass standards (14.3 to 200 kDa) were obtained from GIBCO. Acrylamide and bisacrylamide for SDS-PAGE were from Serva (Heidelberg, Germany), and cellulose thin-layer chromatography aluminum sheets were from Merck (Darmstadt, Germany).

RESULTS

Construction and purification of MBP-EpiD fusion proteins. The epidermin biosynthesis genes *epiA*, *epiB*, *epiC*, and *epiD* are organized as an operon (31). A portion of the operon containing *epiD* is shown in Fig. 1. According to the DNA sequence, *epiD* encodes a 181-amino-acid protein. To obtain more information on its function and biochemical properties, we first tried to express *epiD* in *E. coli* by using the MBP fusion system. By synthesizing specific primers, *epiD* was amplified by the PCR in such a way that the amplified DNA fragment started exactly with the ATG start codon and ended a few base pairs after the TAA stop codon. This fragment was inserted at the single *Stu*I site of the pIH902 polylinker. In the resulting *malE-epiD* fusion, the *epiD* start codon immediately followed the factor Xa cleavage sequence:

malE ... $(AAC)_n$ GCG GCG ATC GAG GGT AGG - ATG TAT GGA AAA ...
...Ile Glu Gly Arg - **Met Tyr Gly Lys** ...
MBP ... $(Asn)_n$ ... factor Xa cleavage sequence - **EpiD**

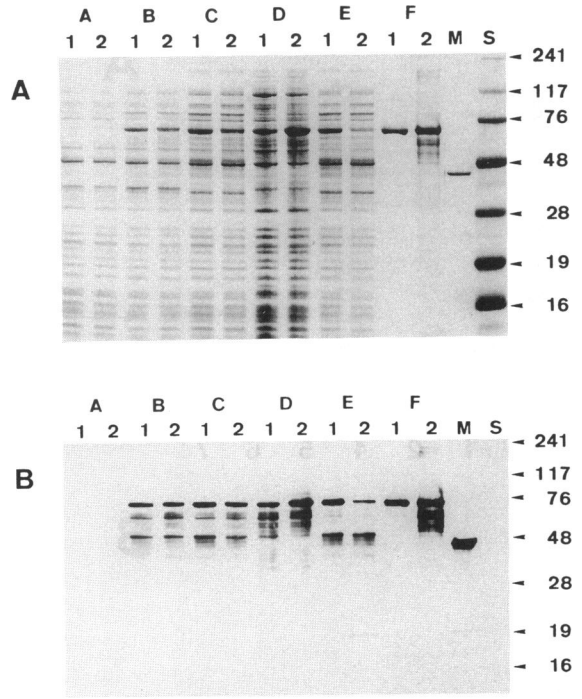

FIG. 2. Identification and localization of the MBP-EpiD and MBP-EpiD* fusion proteins by SDS-PAGE (10% polyacrylamide) and Coomassie staining (A) and by Western blotting with anti-MBP antibodies (B). Lanes: 1, MBP-EpiD (wt); 2, MBP-EpiD*. Sets of lanes: A and B, *E. coli*(pIH902-*epiD*/*epiD**) cells treated with Laemmli buffer before (set A) and after (set B) induction with IPTG; C, crude extract (cells disrupted with glass beads); D, pellet (membrane) fraction; E, soluble proteins; F, after elution from the amylose column; M, 0.4 μg of MBP as a reference protein; S, standard proteins (sizes in kilodaltons are indicated at the right margin). With sets A to E, comparable volumes were applied in lanes 1 and 2; because of the low MBP-EpiD* content in the eluate, the applied volume in lane F2 was 15 times higher than that in lane F1, so that similar amounts (1.2 μg) of MBP-EpiD and MBP-EpiD* were applied.

We took advantage of the destruction of the pIH902 *Stu*I site (AGG/CCT) in this construct by incubating the ligation products with *Stu*I before transformation in *E. coli* TB1; thus, the percentage of transformants with the religated vector was markedly reduced from 99.8% to less than 40%. The fusion protein MBP-EpiD was expressed by 30% of the transformants. The correct orientation and sequence of *epiD* in the *malE-epiD* fusion were determined by DNA sequencing with the *malE*, IH-rev, *epiD-1*, and *epiD-2* primers.

Approximately 90% of the MBP-EpiD fusion protein was found in the soluble protein fraction of *E. coli* TB1(pIH902-*epiD*) cell extracts; there was no evidence that inclusion bodies were formed. The purification and location of the MBP-EpiD fusion protein were followed by SDS-PAGE and protein staining (Fig. 2A, lanes 1). Near the expected size of the fusion protein (65 kDa), a 67-kDa protein was detectable only in cell extracts of IPTG-induced cells. Since this protein reacted with specific anti-MBP antibodies (Fig. 2B) and bound to the amylose resin, it represented the fusion protein. Anti-MBP antibodies also reacted with smaller proteins, which were very likely degradation products of the fusion protein. In uninduced cells, no MBP-specific immune reactive protein of any size was detectable. The 67-kDa MBP-EpiD fusion protein was purified to homogeneity in one step by amylose affinity chromatography (Figs. 2A and B, lanes F1). The purified fusion protein was used to raise polyclonal antibodies directed against EpiD.

The MBP-EpiD fusion protein was yellow and identified as a flavoprotein by its absorption spectrum with maxima at 277, 378, and 449 nm (36) (see Fig. 6B). The coenzyme was very tightly but not covalently attached. It could only be removed by trichloroacetic acid extraction of the fusion protein and was identified by thin-layer chromatography as FMN.

Characterization of *epiD and its gene product.** *S. epidermidis* Tü3298/EMS11 carries a mutation within *epiD* (4), designated *epiD**, which was amplified by the PCR with purified pTü32 from the mutant as a template. This DNA fragment was inserted in the *Stu*I site of the pIH902 polylinker by the procedure described above for the *malE-epiD* fusion. Plasmids were isolated from four *E. coli* TB1 (pIH902-*epiD**) clones, and the entire *epiD** region was sequenced. The *epiD** start codon immediately followed the factor Xa cleavage sequence, and all four isolated plasmids had a point mutation in codon 93, substituting the wild-type GQT (Gly) with GAT (Asp) in *epiD**. This G-to-A transition concurs with ethyl methanesulfonate as the mutagenizing agent (4).

Production of MBP-EpiD* was followed by SDS-PAGE (Fig. 2A, lanes 2) and Western blotting (Fig. 2B, lanes 2). Most of MBP-EpiD* was found in the pellet fraction; the soluble protein fraction contained only 5 to 10% of the entire amount. This is in contrast to MBP-EpiD, most of which was found in the soluble protein fraction (compare lanes E1 and E2 of Fig. 2A). It is very likely that inclusion bodies containing the MBP-EpiD* fusion were formed in *E. coli*. After amylose affinity chromatography, various immune reactive degradation products could be seen (Fig. 2A and B, lanes F2); these results may indicate that MBP-EpiD* is more sensitive than the wild-type fusion protein to proteolytic degradation.

However, the most striking difference was that MBP-EpiD* was not yellow and did not exhibit the characteristic absorption maxima of MBP-EpiD (see above and spectra 2 and 3 of Fig. 6B) when similar concentrations of fusion proteins were used. For this comparison, it was necessary to dilute MBP-EpiD because of the small amount of soluble MBP-EpiD*. The pellet fraction was not yellow either. MBP-EpiD* was unable to bind FMN. Since MBP-EpiD* differed from MBP-EpiD only by the substitution of Gly-93 by Asp, Gly-93 may be important for FMN binding.

Expression of *epiD* in *E. coli* with the T7 promoter-polymerase system. It was previously demonstrated that the 3.1-kb *Hin*dIII-*Pvu*II fragment of pTü32 complements both *epiC* and *epiD* mutants of *S. epidermidis* Tü3298, whereas the 1.7-kb *Eco*RI-*Bam*HI fragment (Fig. 1) only complements the *epiD* mutant (4). For gene expression studies, the 3.1-kb fragment containing *epiC* and *epiD* in the same orientation and *epiQ* and a portion of *epiP* in the opposite orientation was inserted by blunt-end ligation into the *Sma*I site of the T7 promoter-polymerase vector pT7-5 (pT7-5*epiCD*); the smaller fragment, in which most of *epiC* and *epiP* is deleted, was cloned in pT7-5 by *Eco*RI-*Bam*HI ligation (pT7-5*epiD*). The correct orientation of the fragments (T7 promoter in front of *epiCD*) was checked by restriction analysis.

The *E. coli*-specific RNA polymerase was inhibited by rifampin, and the T7 promoter-controlled genes were preferentially expressed after heat induction. Expressed proteins were labeled with [^{35}S]methionine, separated by SDS-

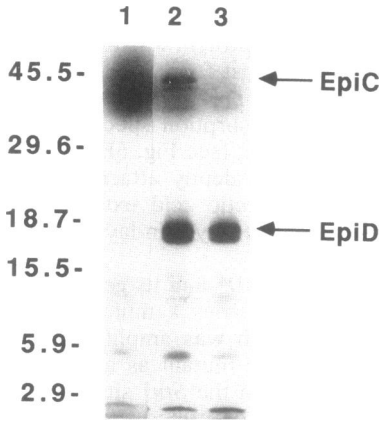

FIG. 3. Autoradiograph of [^{35}S]methionine-labeled EpiC and EpiD expressed with the T7 system in *E. coli* K38 containing plasmids pGP1-2 and the following indicated plasmid (lanes): 1, pT7-5 (negative control); 2, pT7-5*epiCD*; 3, pT7-5*epiD*. *E. coli* RNA polymerase was inhibited by rifampin. Proteins were electrophoresed by Tricine–SDS–15% PAGE. Molecular size markers (kilodaltons) are indicated on the left.

PAGE, and visualized by autoradiography (Fig. 3). In the presence of plasmid pT7-5*epiCD*, a major protein band at 44 kDa and two very narrow protein bands at 18 and 17 kDa were visible. The 44-kDa protein very likely represents EpiC. First, the molecular weight is similar to the expected value of 48,000; second, in the absence of *epiC*, this protein band and two other smaller ones (very likely degradation products) are absent (Fig. 3, lane 3). Since the theoretical molecular mass of EpiD is 20.8 kDa, we assume that the 17- and 18-kDa proteins very likely represent EpiD. Potential explanations for the two migrating forms of EpiD are discussed below.

Comparison of *epiD* expression in *E. coli* with that in *S. epidermidis* Tü3298. Whole-cell lysates of uninduced and induced *E. coli* clones and cell extracts of *S. epidermidis* Tü3298 were analyzed by SDS-PAGE, and EpiD was detected by Western blotting with anti-MBP-EpiD antiserum (Fig. 4). Based on densitometric comparison of the signal intensities of lane A3 [crude extract of *E. coli*(pGP1-2, pT7-5*epiD*)] with those of lane C (crude extract of *S. epidermidis* Tü3298), the concentration of EpiD was approximately 50-fold higher in the induced *E. coli* clones than in *S. epidermidis* Tü3298.

EpiD was poorly cleaved from MBP by factor Xa; less than 3% of the fusion protein was cleaved in 20 h (data not shown). Therefore, this method was not used to obtain purified EpiD. The separated EpiD reacted with anti-MBP-EpiD antiserum (Fig. 4, lane B2), as did the separated MBP.

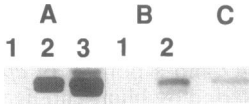

FIG. 4. Comparison of EpiD content in cell extracts of *E. coli* clones and *S. epidermidis* Tü3298 by Western blotting with anti-MBP-EpiD antiserum. Lanes: A1, uninduced *E. coli* K38(pGP1-2, pT-5*epiD*) cells treated with Laemmli buffer; A2, induced cells; A3, induced *E. coli* K38(pGP1-2, pT7-5*epiD*) cells disrupted with glass beads (25 μg of total protein); B1, approximately 5 μg of purified MBP-EpiD fusion protein before factor Xa cleavage (MBP-EpiD and MBP are not shown); B2, MBP-EpiD after factor Xa cleavage (15 h at 25°C; 30 μg of MBP-EpiD per μg of factor Xa); C, crude extract of *S. epidermidis* Tü3298 after disruption with glass beads (30 μg of total protein).

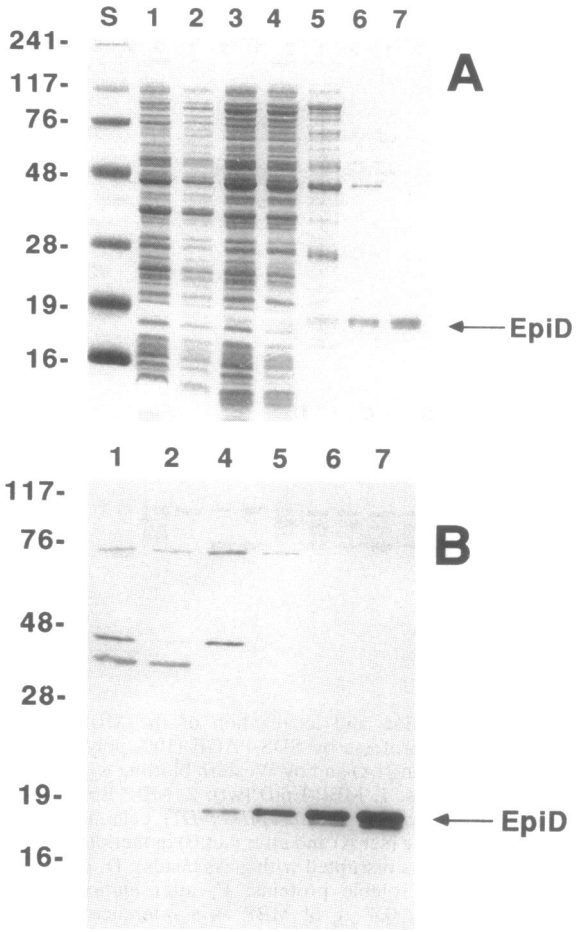

FIG. 5. Monitoring EpiD purification steps by SDS-PAGE (A) and Western blotting with anti-MBP-EpiD antiserum (B). Lanes: 1 and 2, whole-cell lysate of *E. coli*(pGP1-2, pT7-5*epiD*) before (lane 1) and after (lane 2) induction by temperature shift to 42°C; 3, cell extract; 4, supernatant after 150,000 × *g* centrifugation; 5, DEAE pool; 6, Mono-Q pool; 7, Phenylsuperose pool; S, protein standards (sizes in kilodaltons in left margin). Comparable sample volumes were applied in lanes 3 to 7.

Purification of EpiD from *E. coli*(pGP1-2, pT7-5*epiD*). EpiD was isolated from the soluble protein fraction of *E. coli*(pGP1-2, pT7-5*epiD*). It was purified to homogeneity in three chromatographic steps on DEAE-Sepharose Fast Flow, Mono-Q, and Phenylsuperose columns. In both anion-exchange chromatographies, EpiD eluted at 300 mM NaCl; with Phenylsuperose chromatography, EpiD eluted at 0 M ammonium sulfate. From a 2.5-liter culture, 39.5 mg of soluble protein was obtained after cell disruption and ultracentrifugation. After Phenylsuperose chromatography, 0.1 mg of purified EpiD was recovered. Since approximately 75% of EpiD was lost in the course of purification, EpiD composes approximately 1% of the total soluble proteins. The various purification steps were followed by SDS-PAGE (Fig. 5A) and immunoblotting with anti-MBP-EpiD antiserum (Fig. 5B). Here, too, purified EpiD migrated as a double band. All column chromatographic steps were monitored at A_{280} and A_{450}. The EpiD content in the various fractions was always directly proportional to A_{450} (data not shown).

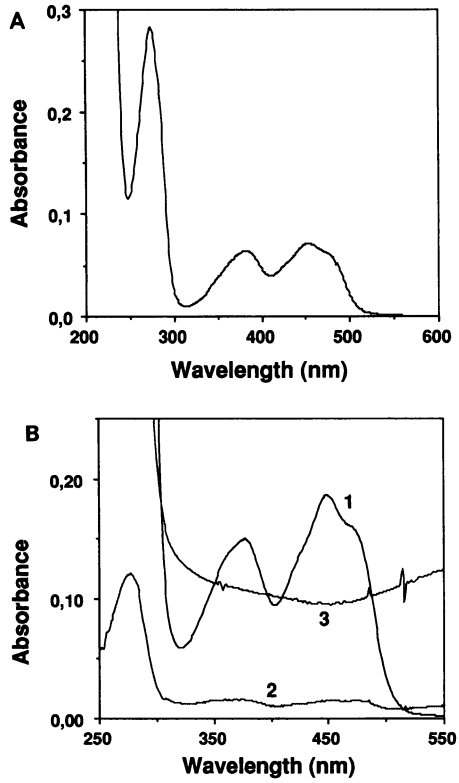

FIG. 6. Absorption spectra of purified proteins. (A) Purified EpiD (100 μg/ml; A_{274}/A_{453} = 3.9). (B) Curves: 1, purified and undiluted MBP-EpiD (680 μg/ml; A_{277}/A_{449} = 10.0); 2, purified and 1:15 diluted MBP-EpiD (45 μg/ml) (the concentration of the fusion protein was adjusted to the concentration of MBP-EpiD* as described in the legend to Fig. 2); 3, MBP-EpiD*.

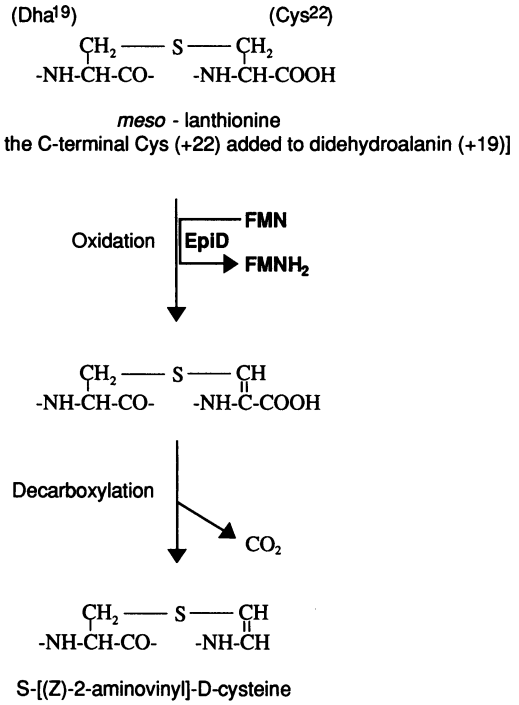

FIG. 7. Model of the C-terminal modification of epidermin and the role of flavoprotein EpiD.

Characterization of purified EpiD. The first 18 N-terminal amino acids of purified EpiD were determined by Edman degradation. The amino acid sequence correlated exactly with the postulated amino acids (Met-Tyr-Gly-Lys...) deduced from the *epiD* sequence (31). The results of the Edman degradation did not reveal any heterogeneity.

Purified EpiD was yellow. The absorption spectrum exhibited maxima at 274, 382, and 453 nm; these maxima are characteristic for flavoproteins in the oxidized state (36) (Fig. 6A). The coenzyme was not covalently bound to EpiD; it was possible to remove it by heat treatment. The liberated flavin component was analyzed by thin-layer chromatography and compared with FMN, flavin adenine dinucleotide, and riboflavin; the isolated coenzyme and FMN were indistinguishable. The molar relationship of FMN and protein was calculated to be 0.7, indicating that, in general, 1 molecule of FMN is bound to 1 molecule of EpiD.

DISCUSSION

In SDS-PAGE, the 181-amino-acid EpiD migrated as 18- and 17-kDa proteins in preparations from both *E. coli*(pGP1-2, pT7-5*epiD*) and *S. epidermidis* Tü3298. A simple explanation is that the 17-kDa form is generated by proteolytic degradation of the 18-kDa protein. In this case, both microorganisms must possess a very similar-acting protease. The results of N-terminal Edman degradation of a purified EpiD preparation, consisting of approximately 30% of the 17-kDa form, were very homogeneous (less than 2% impurity), indicating that the N termini of the 18- and 17-kDa forms are identical. This does not rule out the possibility of proteolytic cleavage at the C terminus; however, other explanations are possible. EpiD contains four cysteine residues; disulfide bridges could form. Although SDS-PAGE was always performed under reducing conditions, a portion of EpiD might have become oxidized during electrophoresis, thus causing a different electrophoretic mobility. To explore these possibilities, the two forms are presently being separated and analyzed.

EpiD is a rather hydrophobic protein, composed of 41% hydrophobic residues (31) and distinguished by its strong interaction with a hydrophobic matrix, such as Phenylsuperose. We assume that epidermin modification is catalyzed by a membrane-associated multienzyme complex; the hydrophobic character of EpiD could thus be important for protein-protein interactions.

This study shows that EpiD is a flavoprotein with FMN as a coenzyme. Since flavin coenzymes are normally involved in oxidation-reduction reactions, EpiD belongs to the class of oxidoreductases. When we compare the primary amino acid sequence of epidermin (33) with the chemical structure of the mature form (1), there is only one obvious reaction that requires an oxidoreductase activity: the removal of two reducing equivalents from a —C—C— group to form a —C=C— group. Such a desaturating reaction takes place at the last cysteine residue of the epidermin C terminus. The postulated modification reaction catalyzed by EpiD is illustrated in Fig. 7. The C termini of the lantibiotics epidermin, gallidermin (18), and mersacidin (11) are distinguished by a ring structure in which *S*-(2-aminovinyl)-D-cysteine represents a characteristic building block. The biochemical synthesis of *S*-[(*Z*)-2-aminovinyl]-D-cysteine may proceed in four steps as follows: (i) serine in position +19 of the primary translational product is dehydrated, forming didehydroala-

nine (the removal of water is accompanied by double-bond formation); (ii) the thiol group of the C-terminal cysteine, Cys-22, is linked to the double bond of the didehydroalanine at position 19, forming the C-terminal *meso*-lanthionine; (iii) two reducing equivalents are removed from the cysteine residue of the C-terminal *meso*-lanthionine, forming a —C=C— double bond (this reaction is very likely catalyzed by EpiD); (iv) removal of the C-terminal carboxyl group by a decarboxylase reaction catalyzed by a separate enzyme or occurring spontaneously.

With respect to the order of the modification reactions, we assume that the four lanthionine rings are formed first and then the C-terminal lanthionine is oxidized by EpiD. In the course of the oxidation, EpiD-FMN becomes reduced. How the enzyme becomes reoxidized is unknown. According to this hypothesis, EpiD very likely catalyzes the final step in the modification of pre-epidermin and its reaction product is then processed by the leader peptidase, forming mature epidermin.

The *epiD* mutant *S. epidermidis* Tü3298/EMS11 is unable to synthesize active epidermin. When *epiD**, cloned and expressed by using the *malE* fusion system in *E. coli*, was sequenced, only a point mutation causing a substitution of Gly-93 by Asp was found, leading to the inability of MBP-EpiD* to bind FMN. We conclude that EpiD* is also unable to bind FMN; this is a convincing explanation for the inability of *S. epidermidis* Tü3298/EMS11 to produce epidermin.

There are two molecular explanations for the inability of MBP-EpiD* to bind FMN: (i) the sequence -Lys-Ile-Ala-Asn-Gly-93-Ile-Cys-Asp- is part of the FMN binding site or (ii) Gly-93 is outside the FMN binding site, but its substitution by Asp causes a conformational change in the protein because the additional negative charge leads to detrimental effects on FMN binding. For example, the mutation may prevent disulfide bridging of the nearby cysteine residue and, as a consequence, may lead to a conformational change. Evidence for a profound effect of the mutation on the tertiary structure is the increased insolubility of MBP-EpiD* as compared with that of MBP-EpiD.

We are presently attempting to purify the epidermin precursor from the mutant *S. epidermidis* Tü3298/EMS11 to use as a substrate for EpiD in the analysis of our model of EpiD function in epidermin biosynthesis. This proposed reaction of EpiD is a completely new posttranslational modification reaction.

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