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Determination of the Gene Sequence and the Molecular Structure of the Enterococcal Peptide Antibiotic AS-48

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The structural gene of the enterococcal peptide antibiotic AS-48 (*as-48*) has been identified and cloned by using two degenerate 17-mer DNA oligonucleotides on the basis of the amino acid sequences of two peptides obtained by digestion of the antibiotic with Glu-C endoproteinase. That *as-48* gene codes for a 105-amino-acid prepeptide, giving rise to a 70-amino-acid mature protein. Comparative analysis demonstrated that the 16-amino-acid sequence of one of the AS-48 Glu-C peptides, designated V8-5, was composed of a 12-amino-acid sequence corresponding to the C-terminal end sequence (from isoleucine +59 to tryptophan +70 [I^{+59} to W^{+70}]) of the prepeptide and terminated in four residues forming the N terminus (M^{+1} to E^{+4}) of a putative AS-48 propeptide. These data, combined with the characteristics of the gene sequence, strongly suggested that the antibiotic peptide was a 70-residue cyclic molecule. We propose that the AS-48 translated primary product is very likely submitted to a posttranslational modification during secretion (i) by an atypical or a typical signal peptidase that cleaves off a 35-residue or shorter signal peptide, respectively, from the prepeptide molecule and (ii) by the linkage of the methionine residue (M^{+1}) to the C-terminal tryptophan residue (W^{+70}) to obtain the cyclic peptide (a tail-head linkage).

For many years, it has been known that enterococci produce inhibitory substances such as bacteriocins and hemolysins that are encoded by large widely disseminated and transmissible plasmids (4, 9, 13, 20, 22). These plasmids usually are able to transfer to recipient cells at a relatively high frequency in broth (4, 13, 24, 25). The best-characterized bacteriocin produced by enterococci is the bacteriocin-hemolysin encoded by the sex pheromone plasmid pAD1 (11, 14). The cytolytic system of pAD1 revealed two protein components, designated A (activator) and L (lytic), able to complement each other extracellularly (11, 14) and to contribute to bacterial virulence in an animal model (15).

AS-48 is a plasmid-encoded peptide antibiotic produced by *Enterococcus faecalis* S-48 with broad antimicrobial activities against gram-positive and gram-negative bacteria (6). Some pathogenic bacteria, including *Staphylococcus*, *Enterococcus*, and *Salmonella* species, are highly sensitive to AS-48 (7). Insertion of the peptide into the cytoplasmic membrane of target cells or artificial membranes renders the membrane permeable to small molecules (ions, amino acids), causing the release of cytoplasmic material and the lysis of sensitive cells (8).

Because of its strongly cationic properties and biological activity, the AS-48 peptide antibiotic could be related to the group of bacteriocins and peptide antibiotics, mainly produced by lactic acid bacteria, which appear to function by permeabilizing the cytoplasmic membrane. They include lactococcins A, B, and G (12, 27, 39); lactocin S (26); and the group of lantibiotics, e.g., nisin (2, 10), epidermin (34), Pep 5 (19), lacticin 481 (30), and subtilin (35), which contain several unusual amino acids such as lanthionine, β-methyllanthionine, dehydroalanine, and dehydrobutyrine (16–18). Nevertheless, AS-48 should not be included in the lantibiotic group, because AS-48 does not contain lanthionine or other unusual amino acids (6).

We now report the cloning and sequencing of the structural *as-48* gene of the antibiotic AS-48 and show that a posttranslational modification must occur to produce a cyclic molecule. Amino acid sequence determination provided evidence for the existence of a tail-head bond allowing the cyclization of the molecule. This appears to be the first example of a new type of ribosomally synthesized cyclic peptide antibiotic.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. All enterococcal strains were grown at 37°C in brain heart infusion broth and maintained as frozen stocks at −20°C in brain heart infusion broth containing 20% glycerol. *E. faecalis* JH2-2(pMB2) was obtained by conjugal transfer, in broth, of the AS-48 determinant from *E. faecalis* A-48-32 (23, 24) to the plasmid-free strain JH2-2 (40). Transconjugants were selected on brain heart infusion agar plates containing rifampin (25 μg/ml) and tested for the ability to produce AS-48. Typically, AS-48 was transferred at a frequency of 10^{-4} to 10^{-3} per donor. *Escherichia coli* HB101 was used for the propagation of plasmid pUC18 (41) in Luria-Bertani broth (32) with ampicillin (50 μg/ml) added.

Enzymes and reagents. Lysozyme, restriction endonucleases, T4 DNA ligase, Klenow fragment of DNA polymerase I, and molecular weight standards were obtained from Boehringer Mannheim (Mannheim, Germany). Universal and reverse sequencing primers were purchased from Pharmacia. RNase A was from Sigma Chemical Co., and [^{35}S]dATP was from Amersham Corp. A T7 polymerase kit for DNA sequencing was from Pharmacia.

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Isolation of AS-48 peptide fragments and amino acid sequencing. After purification, 30 nmol of the AS-48 protein was digested with the endoproteinase Glu-C isolated from *Staphylococcus aureus* V8 (Boehringer Mannheim). A 1/30 enzyme/substrate ratio was used in 20 mM NaH_2PO_4 (pH 7.0) with a 5-h incubation at 37°C. The peptides resulting from this digestion were subjected to reversed-phase high-performance liquid chromatography on a 5-μm C_{18} column (2.1 by 220 mm) (Brownlee; ABI, Foster City, Calif.). The gradient was formed with a 140A solvent delivery system (ABI). The composition of solvent A was 0.1% trifluoracetic acid in Milli Q water (Millipore, Bedford, Mass.). Solvent B was 0.1% trifluoroacetic acid in 70% acetonitrile–water. Detection of the peptides was made with a diode array detector (model 1000S; ABI), and the A_{220} was registered on a paper recorder (speed, 0.5 cm/min). Fractions were collected manually in polypropylene tubes and dried in a Speed Vac concentrator (Savant, Hicksville, N.Y.). Prior to sequence analysis, peptides were redissolved in 0.1% trifluoroacetic acid–water. The remaining peptide solutions were stored at −18°C.

Sequence analysis was performed with a pulsed liquid-phase sequenator (model 477A or 476A; ABI) with on-line phenylthiohydantoin analysis on the 120A analyzer (ABI). Sequencing reagents and solvents were obtained from ABI.

DNA isolation, analysis, and manipulations. Plasmid DNAs were isolated from *E. faecalis* strains as described by Anderson and McKay (1) and purified by CsCl isopycnic centrifugation (32). Small- and large-scale isolation of plasmids from *E. coli* was performed by the alkaline lysis method (32). Restriction fragments of the desired size for cloning were separated on 0.7% agarose gels, isolated, and purified with GeneClean (Bio 101, La Jolla, Calif.).

General procedures for cloning and DNA manipulations were essentially as described by Sambrook et al. (32).

Nucleic acid hybridizations and nucleotide sequencing. On the basis of the sequences of the AS-48 peptides V8-5 (IKKKGKRAVIAWMAKE) and V8-7 (FGIPAAVAGTVL NVVE), two degenerate oligodeoxynucleotide probes, oligo-V8-5 [5′ AT(T/C/A) AA(A/G) AA(A/G) AA(A/G) GC(T/C/A/G) AA 3′] and oligo-V8-7C [5′ TC (A/G/T/C)AC (A/G/T/C)AC (A/G)TT (A/G/T/C)A(G/A) (A/G/T/C)AC-3′], were synthesized by Isogen (Amsterdam, The Netherlands). These probes were labeled in 3′ with digoxigenin-11–ddUTP by using terminal transferase according to the manufacturer's instructions (Boehringer Mannheim). Southern blots were made by transfer of restriction endonuclease-digested pMB2 plasmid on 0.7% agarose gels to Nytran membranes (Schleicher & Schuell) (38). Hybridization and detection of the probes were performed according to the manufacturer's instructions (Boehringer Mannheim).

Nucleotide sequencing by the dideoxynucleotide method (33) was carried out with restriction fragments cloned into pUC18 by using universal, reverse, and synthetic primers.

DNA sequences were compiled with the MAP program from the Genetics Computer Group package (5). The GenBank, translated GenBank, and National Biomedical Research Foundation sequence data banks were searched for similar DNA or amino acid sequences by using the FASTA and TFASTA programs of Pearson (29) on a Vax computer. The secondary structure was predicted by using the Chou and Fasman method (3). Hydropathicity was plotted by the Kyte and Doolittle method (21).

Nucleotide sequence accession number. The EMBL accession number for the nucleotide sequence shown in Fig. 3 is X79542.

TABLE 1. N-terminal amino acid sequences of peptides obtained after fragmentation of AS-48 with Glu-C endoproteinase

Peptide	Amino acid sequence	Positions[a]
V8-4	SIKAYLKKE	50–58
V8-5	IKKKGKRAVIAWMAKE	59–4
V8-7	FGIPAAVAGTVLNVVE	5–20

[a] The positions of the sequenced parts of the peptides are indicated in Fig. 3.

RESULTS

Peptide sequences of AS-48. The purification procedure of the AS-48 peptide was described previously (6). Attempts to determine the sequence of AS-48 by Edman degradation were unsuccessful. This suggested that AS-48 amino-terminal and carboxyl groups were blocked or, alternatively, that the peptide had a cyclic structure. In addition, efforts to digest AS-48 with carboxypeptidases A and B were unsuccessful (data not shown). Digestion of pure AS-48 with V8 endopeptidase and determination of the sequences of the purified peptides by Edman degradation gave a partial amino acid sequence of the antibiotic. The sequences of these peptides are shown in Table 1. These results confirmed that no unusual amino acids were present in these peptide fragments.

Location of the structural gene *as-48*. It was demonstrated previously (23, 24) that a 68-kb plasmid, pMB2, from *E. faecalis* S-48 bore the genetic determinant for the AS-48 character (production of and immunity to AS-48 antibiotic). Transfer of plasmid pMB2 by conjugation from *E. faecalis* A-48-32 into the plasmid-free strain JH2-2 facilitated its characterization (data not shown). The construction of a restriction map of plasmid pMB2 was achieved by using simple and double restrictions with *Bam*HI, *Bgl*II, *Pst*I, *Sal*I, *Sac*I, *Sma*I, and *Xba*I. One of these enzymes, *Sma*I, linearized the plasmid pMB2 and was considered to be the reference site for the other enzymes (Fig. 1 and 2).

Two oligonucleotide probes deduced from the amino acid sequences of peptides V8-5 and V8-7C (see Materials and Methods) were used to localize the *as-48* structural gene. Both oligonucleotides hybridized specifically with the same restriction fragments from plasmid pMB2 (Fig. 1 and 2). The 3.9-kb fragment of *Bgl*II-digested plasmid pMB2 that hybridized with

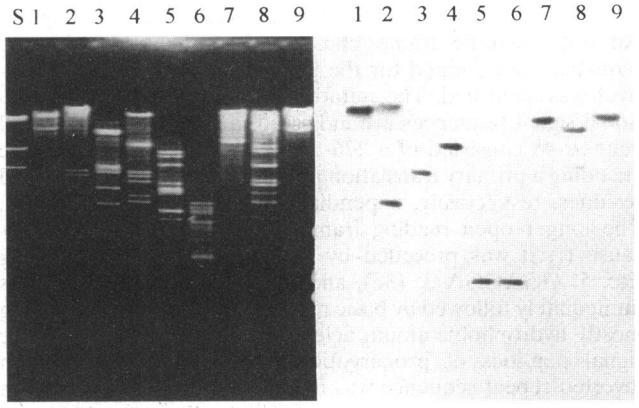

FIG. 1. Agarose gel of digested *E. faecalis* pMB2 plasmid DNA (left panel) and hybridization of digested plasmid DNA with a wobbled 17-mer oligonucleotide, V8-5 (right panel). This probe was derived from the expected propeptide sequence of AS-48. Lanes: S, molecular weight standard lambda *Hin*dIII; 1, *Bam*HI; 2; *Bgl*II; 3, *Eco*RI; 4, *Eco*RV; 5, *Hin*dIII; 6, *Hin*cII; 7, *Kpn*I; 8, *Pst*I; 9, *Sac*I.

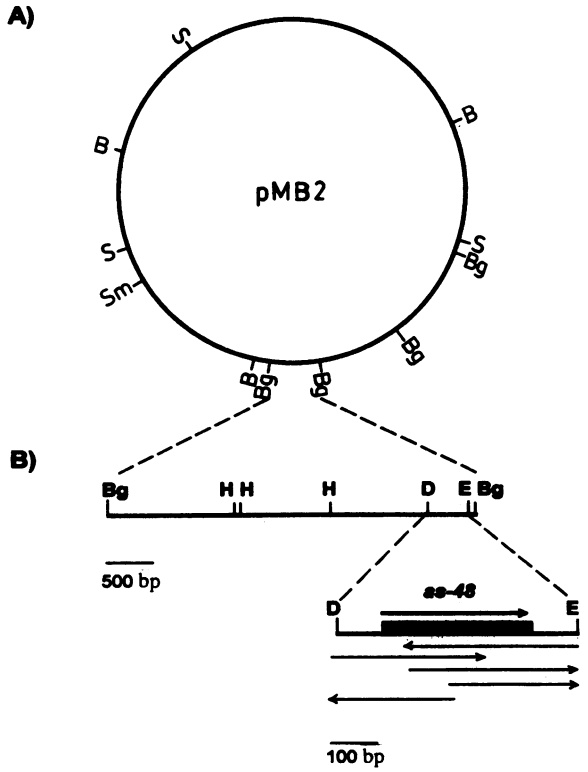

FIG. 2. Restriction map (A) and sequencing strategy (B) of the pMB2 region containing the *as-48* gene. A 3.9-kb *Bgl*II (Bg) fragment obtained from pMB2 plasmid was subcloned into the pUC18 plasmid, yielding the recombinant plasmid pMB222. An *Eco*RI-*Dde*I (E-D) fragment from pMB222 plasmid was sequenced in both orientations. The nucleotide sequence was determined by the dideoxy method according to the strategy shown. The sequence obtained is shown in Fig. 3. Bg, *Bgl*II; H, *Hin*dIII; B, *Bam*HI; Sm, *Sma*I; S, *Sac*I.

```
          10                  30                  50
CTAAGAAGTGTCTAGTTATTTTTTCTTGGGTTATTTACAGGAATAGATATGTTATTGCTT
Dde I
          70                  90                  110
GCATCAAAATAAACTACATGGGTATAATAGCAATGAAATGCATTTCAAAAATATTTTGAG

 SD       130                 150                 170
GAGGAGTATCATGGTTAAAGAAAATAAATTTTCTAAGATTTTTATTTTAATGGCTTTGAG
          M  V  K  E  N  K  F  S  K  I  F  I  L  M  A  L  S

          190                 210                 230
TTTTTTGGGGTTAGCCTTGTTTAGTGCAAGTCTTCAGTTTTTGCCGATTGCACATATGGC
 F  L  G  L  A  L  F  S  A  S  L  Q  F  L  P  I  A  H  M  A
                                                      -1 +1

          250                 270                 290
TAAAGAGTTCGGTATACCAGCAGCAGTTGCAGGAACTGTGCTTAATGTAGTTGAAGCTGG
 K  E  F  G  I  P  A  A  V  A  G  T  V  L  N  V  V  E  A  G
                                                         20

          310                 330                 350
TGGATGGGTCACTACTATTGTATCAATTCTTACTGCTGTAGGTAGCGGAGGTCTTTCTTT
 G  W  V  T  T  I  V  S  I  L  T  A  V  G  S  G  G  L  S  L
                                                         40

          370                 390                 410
ACTCGCTGCAGCAGGAAGAGAGTCAATTAAAGCATACCTTAAGAAAGAAATTAAGAAAAA
 L  A  A  A  G  R  E  S  I  K  A  Y  L  K  K  E  I  K  K  K
                                                         60

          430                 450                 470
AGGAAAAAGAGCAGTTATTGCTTGGTAATTTAACAATATGATAAAAAAACAGGATATTTT
 G  K  R  A  V  I  A  W  *
                     70

          490                 510                 530
CTAGAGATATTCTGTTTTTTAATTAAAAAAGGGGGGCGCTCATGAATCTCTTTGGAATTC
                                                      Eco RI
```

FIG. 3. Sequence of a portion of a 3.9-kb *Bgl*II fragment from the pMB2 plasmid that contains the gene encoding the precursor peptide AS-48. The peptide portion in boldface corresponds to the potential leader region. The sequence of the mature region is numbered from 1 to 70. The putative ribosome binding site (Shine-Dalgarno sequence) encompassing 6 bp is underlined, labeled SD, and is typical of those observed in *E. coli*. The inverted repeats acting as a putative terminator sequence are also underlined; this sequence has a free energy of −7.4 kcal (−31 kJ)/mol.

both probes was inserted into pUC18 and cloned in *E. coli*, yielding a recombinant plasmid designated pMB222 (Fig. 2).

DNA sequence of the region containing the structural gene of peptide AS-48. A much better localization of the AS-48 structural gene was achieved by using double and triple restrictions of plasmid pMB222 (data not shown). The 540-bp *Eco*RI-*Dde*I fragment from pMB222 was sequenced (Fig. 3). An open reading frame that exhibited identities with the sequences determined for the purified AS-48 peptide derivatives was identified. The putative transcriptional and translational signal sequences are indicated in Fig. 3. The structural gene *as-48* consisted of a 276- or 315-bp open reading frame encoding a primary translation product of 92 or 105 amino acid residues, respectively, depending on the initiation site used. The longer open reading frame seemed more probable because (i) it was preceded by a potential ribosome binding site, 5′ GGAGGA 3′ (36), and (ii) the first methionine was immediately followed by basic residues and a region containing mostly hydrophobic amino acids. These features characterize signal peptides of prokaryotic secreted proteins (31). An inverted repeat sequence was identified 14 bases downstream from the TAA termination codon. This sequence could form a stem-loop structure having an estimated free energy of −7.4 kcal (−31 kJ)/mol and could serve as a transcription terminator signal of *as-48*.

A comparison of the amino acid sequence obtained from the *as-48* gene with the partial peptide sequence of AS-48 (Table 1) demonstrated that the 16-amino-acid peptide V8-5 was formed of a 12-amino-acid sequence corresponding to the C-terminal end of AS-48 prepeptide, immediately followed by 4 amino acids found at positions 36 to 39 of the prepeptide. Hence, it appeared that AS-48 was synthesized as a 105-amino-acid prepeptide that was processed between His at -1 (H^{-1}) and Met at $+1$ (M^{+1}) to produce a 70-amino-acid propeptide. These results also clearly suggested that a tail-head linkage of the AS-48 propeptide must have occurred during maturation to yield the cyclic peptide antibiotic AS-48.

The hydropathy analysis profile with Kyte and Doolittle parameters (21) indicated that the leader sequence and the propeptide were mostly hydrophobic, except for the 18 C-terminal residues and the first N-terminal residues that formed mainly hydrophilic regions. The putative cleavage site of the leader signal was also hydrophilic (Fig. 4A).

A search of protein and DNA databases by using the FASTA and TFASTA programs and comparison of AS-48 with other previously sequenced bacteriocins and peptide antibiotics revealed no significant homologies.

DISCUSSION

As reported previously (6), *E. faecalis* S-48 produces a peptide antibiotic designated AS-48, which was shown to be encoded by the 68-kb conjugative plasmid pMB2 (24). The work presented here describes the identification and molecular analysis of the genetic determinant of AS-48. The biological

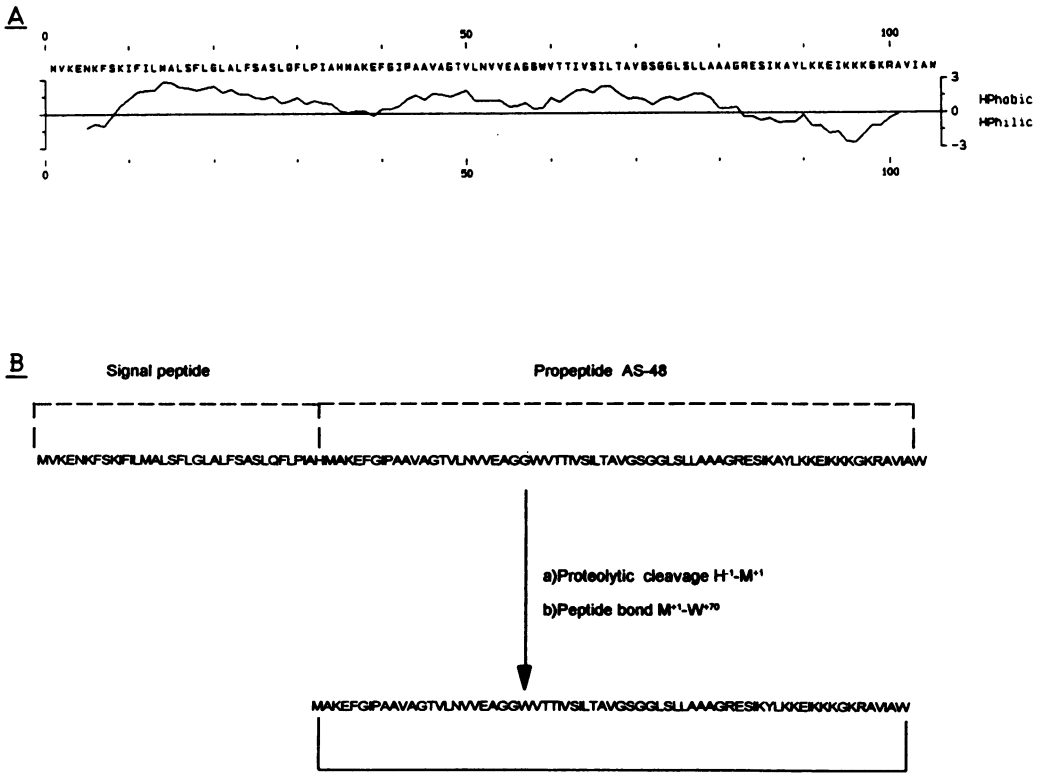

FIG. 4. Prediction plots for prepeptide AS-48. (A) Hydrophobic (HPhobic) profile of prepeptide AS-48. (B) Hypothetical maturation procedure for AS-48 peptide antibiotic. First, there is a cleavage reaction between His^{-1} and Met^{+1}. Second, there is a water elimination reaction between M^{+1} and W^{+70}, yielding a cyclic peptide linked tail to head. HPhilic, hydrophilic.

properties of AS-48 are characteristic of the particular family of low-molecular-mass antibiotics in the range 2,000 to 8,000 Da, produced mostly by lactic acid bacteria and containing basic and hydrophobic amino acid residues conferring a general cationic and hydrophobic character. They include antibacterial substances such as lactococcins A, B, and G; lactacins B and F; diplococcin; and others (17, 18).

All previous studies have indicated that gram-positive bacteriocins and peptide antibiotics belonging to that specific class are usually synthesized as prepeptide and then are submitted to posttranslational enzymatic modifications of the propeptide region prior to signal peptide cleavage (17, 18). These enzymatic modifications result in the formation of novel amino acids such as lanthionine, β-methyllanthionine, and dehydroalanine (16–18). The active molecule, AS-48, is apparently likewise processed from a longer primary translated product. But, in terms of its primary structure, it is clearly different from those other peptide antibiotics. In addition, it is not submitted to structural modification of its amino acids. Several observations support these facts. The mature AS-48 has an estimated size of about 7,500 Da (6) and contains, at the maximum, 75 amino acids, none of which are unusual (6). However, on the basis of the *as-48* gene sequence, the primary product is a 105-amino-acid-long polypeptide whose N-terminal first 35 amino acids could form a long signal peptide. A typical ribosome binding site (GGAGGA) preceding the 5′ end of the open reading frame confirms this hypothesis. Finally, downstream from the TAA stop codon, a 17-bp inverted repeat could serve as a transcription terminator.

The comparative analysis of the amino acid sequence deduced from *as-48* gene and that of the AS-48 peptide V8-5 (a fragment obtained by Glu-C endoproteinase digestion of AS-48) demonstrates unequivocally that the mature peptide AS-48 has a cyclic structure. The 16-amino-acid peptide V8-5 starts with a 12-amino-acid sequence corresponding to the C-terminal end sequence (I^{+59} to W^{+70}) of the translated gene product (Table 1) and terminates with four residues (MAKE) that would be close to the N terminus of the propeptide AS-48. This suggests that the sequence M^{-35} to H^{-1} (Fig. 4B) is a signal peptide of the primary product of the *as-48* gene. Its structural features are similar to those of other signal peptides previously described (31, 37). There is, indeed, a positively charged NH_2 terminus (four residues) followed by a stretch of hydrophobic residues and a polar region next to the cleavage site. However, in comparison with those signal peptides, the AS-48 leader sequence would have an atypical histidine residue at position −1 of the cleavage site. At this stage, one cannot exclude that a typical signal peptidase is implicated in the processing of the AS-48 prepeptide. In that case, the propeptide would be longer by one (H^{-1}) or more amino acids than the propeptide shown in Fig. 4B.

Consequently, the AS-48 translated primary product could undergo the following processing steps: (i) a cleavage of the signal peptide of the prepeptide and (ii) a tail-head linkage of the propeptide molecule to yield the cyclic mature peptide. This structure needs a water elimination reaction between M^{+1} and W^{+70} during the peptide bond formation. Depending on the position of the signal peptide cleavage site, one or two enzymes could be required during the processing. If the cleavage occurs between H^{-1} and M^{+1}, one might speculate that a specific transpeptidase could catalyze the double reaction: a proteolytic cleavage to remove the 35-amino-acid leader

sequence and the linkage of the N-terminal methionine to the C-terminal tryptophan (Fig. 4B). However, if a normal signal peptidase is used and cleaves a peptide bond upstream of the H^{-1} residue, a second enzyme would be needed to remove the propeptide N-terminal amino acid(s) in excess and link the N-terminal M^{+1} with the C-terminal W^{+70}.

The antimicrobial effect of AS-48 is due to its ability to form pores in the membranes of sensitive bacteria, leading to the efflux of small molecules and the depletion of the membrane electrical potential and ultimately leading to cell death (8). The hydrophobicity as well as the strongly cationic charge of AS-48 (Fig. 4A) are two important characteristics shared by lethal peptide antibiotics having the cytoplasmic membrane as the target (28). Secondary structure predictions suggested that a region in AS-48 starting with a glutamic (E^{+20}) residue and ending with glycine (G^{+38}) may form an amphipathic alpha-helix wheel (data not shown). Such amphipathic structures can form oligomers and reorient themselves within the cellular membranes to create pores and ion channels (28). Moreover, the charged amino acid residues of AS-48 were located at the C-terminal end (K^{+52} to R^{+65}) of the propeptide chain (Fig. 4). On this basis, one can propose that the amphipathic region of AS-48 spans the cytoplasmic membrane and participates in the pore formation, while the 14-amino-acid-long polar C-terminal region remains outside of the membrane. That polar region could create a local high concentration of monomers near the cell membrane or, alternatively, might stabilize the correct peptide configuration in a hydrophilic environment.

Finally, the physiological meaning of the production of the cyclic peptide antibiotic AS-48 is not yet clear. It is conceivable that it may be related to resistance to proteolysis or even to its biological activity. More detailed studies of the mechanism of action of both the native and the mature modified AS-48 will be required before the function of this posttranslational modification can be properly understood.

Anyway, we believe that this cyclic AS-48 peptide is the first example of a posttranslational modification of ribosomally synthesized peptides or proteins by tail-head linkage.

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