Enterocins L50A and L50B, Two Novel Bacteriocins from
Enterococcus faecium L50, Are Related to Staphylococcal Hemolysins

LUIS M. CINTAS,1† PILAR CASAUS,1† HELGE HOLO,1 PABLO E. HERNÁNDEZ,2
INGOLF F. NES,1 AND LEIV SIGVE HÅVARSTEIN1*

Laboratory of Microbial Gene Technology, Department of Biotechnological Sciences, Agricultural
University of Norway, N-1432 Ås, Norway,1 and Departamento de Nutrición y Bromatología III,
Facultad de Veterinaria, Universidad Complutense de Madrid, 28040-Madrid, Spain2

Received 10 November 1997/Accepted 17 February 1998

Enterocin L50 (EntL50), initially referred to as pediocin L50 (L. M. Cintas, J. M. Rodríguez, M. F. Fernández, K. Sletten, I. F. Nes, P. E. Hernández, and H. Holo, Appl. Environ. Microbiol. 61:2643–2648, 1995), is a plasmid-encoded broad-spectrum bacteriocin produced by Enterococcus faecium L50. It has previously been purified from the culture supernatant and partly sequenced by Edman degradation. In the present work, the nucleotide sequence of the EntL50 locus was determined, and several putative open reading frames (ORFs) were identified. Unexpectedly, two ORFs were found to encode EntL50-like peptides. These peptides, termed enterocin L50A (EntL50A) and enterocin L50B (EntL50B), have 72% sequence identity and consist of 44 and 43 amino acids, respectively. Interestingly, a comparison of the deduced sequences of EntL50A and EntL50B with the corresponding sequences obtained by Edman degradation shows that these bacteriocins, in contrast to other peptide bacteriocins, are secreted without an N-terminal leader sequence or signal peptide. Expression in vivo and in vitro transcription/translation experiments demonstrated that entL50A and entL50B are the only genes required to obtain antimicrobial activity, strongly indicating that their bacteriocin products are not posttranslationally modified. Both bacteriocins possess antimicrobial activity on their own, with EntL50A being the most active. In addition, when the two bacteriocins were combined, a considerable synergism was observed, especially with certain indicator strains. Even though the enterocins in some respects are similar to class II bacteriocins, several conserved features common to class II bacteriocins are absent from the EntL50 system. The enterocins have more in common with members of a small group of cytolytic peptides secreted by certain staphylococci. We therefore propose that the enterocins L50A and L50B and the staphylococcal cytolsins together constitute a new family of peptide toxins, unrelated to class II bacteriocins, which possess bactericidal and/or hemolytic activity.

Peptide bacteriocins are a heterogeneous group of ribosomally synthesized antimicrobial compounds (31). In recent years, a large number of bacteriocins produced by lactic acid bacteria have been described, but relatively few have been characterized at the molecular level (12, 14, 28, 31, 38, 41). The lantibiotics (class I) are small (1.8- to 3.5-kDa) polycyclic peptides containing posttranslationally modified amino acids, such as α,β-didehydroalanine, α,β-didehydrobutyrylaine, lanthionine, and β-methylthionin (30, 41). Other bacteriocins, the so-called nonlantibiotics (class II), consist of small (<13-kDa), heat-stable, cationic, and hydrophobic peptides containing only unmodified amino acids (31, 32). Both lantibiotics and nonlantibiotics are ribosomally synthesized as precursor peptides containing an N-terminal leader sequence, which is cleaved off concomitantly with export (23). The leader sequences of most nonlantibiotics are cleaved off and some of these are the double-glycine type. They are clearly related and may serve as a recognition signal for the processing and secretion apparatus (22, 52). The removal of double-glycine-type leader sequences and the translocation of bacteriocins across the cytoplasmic membrane are accomplished by dedicated ATP-binding cassette (ABC) transporters and their accessory proteins (23). Some bacteriocins, such as acidocin B (34), divergicin A (58), bacteriocin 31 (50), and enterocin P (7), have recently been shown to be exported by the general secretory pathway (GSP) (40). These GSP-dependent proteins contain a hydrophobic N-terminal extension, called the signal peptide, which consists of a positively charged N terminus, a hydrophobic core, and a cleavage site (18, 27, 54). Signal peptides are removed by a specific signal peptidase during protein translocation across the cytoplasmic membrane (40, 55).

Most bacteriocins secreted into the medium display full antimicrobial activity by themselves (the so-called one-peptide bacteriocins); however, two-peptide bacteriocins require the complementary action of two peptides for full activity. This group includes lactococcin G (39), lactococcin M (51, 53), lactacin F (1), plantaricin S (39), plantaricins EF and JK (3, 13), thermophilin 13 (36), and probably acidocin J132 (49).

Recently, Enterococcus faecium L50 (previously identified as Pediococcus acidilactici L50) has been reported to produce an antimicrobial substance which inhibits growth of a broad spectrum of spoilage and food-borne pathogenic bacteria, including Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus, Clostridium perfringens, and Clostridium botulinum (5, 6, 8). The data from the initial biochemical studies suggested that the antimicrobial activity arises from a single 5,250-Da ther-
mostable peptide consisting of 41 amino acids, whose NH\_2 terminus is blocked for sequencing by Edman degradation (5, 6). However, we show in this work that the antimicrobial activity arises from two highly similar, small, unmodified, cat-
ingonic, and hydrophobic peptides, both closely related to the bacteriocin previously identified as enterocin L50.

### MATERIALS AND METHODS

**Bacterial strains, media, and growth conditions.** *E. faecium* L50, *E. faecium* T136, *Lactobacillus sake* subsp. *cremoris* CNRZ 177, and *Lactococcus lactis* subspp. *cremoris* CNRZ 347 were cultured in MRS broth (Difco, Detroit, Mich.) at 30°C. *Escherichia coli* strains were propagated in Luria-Bertani (LB) broth (40°C) at 37°C with shaking (250 rpm.). Transformants were selected on LB agar (1%) and propagated in LB broth, containing ampicillin (50 μg/mL) and/or chlorampheni-

**Antimicrobial activity assays.** Cell-free supernatants of *E. faecium* L50 and *E. coli* BL21 (DE3)(pLysS) (Novagen Inc., Madison, Wis.) were obtained by centrifugation at 12,000 × g for 10 min at 4°C and subsequent filter sterilization. Antimicrobial activity was assayed by spotting 5 μL of supernatants or 2 μL of the in vitro-synthesized peptides EntL50A and/or EntL50B onto MRS agar (1.5%) plates previously seeded with 10⁶ CFU of a fresh overnight culture of *P. acidilacti-

**Plasmid DNA isolation and sequencing.** Plasmid DNA of *E. faecium* L50 was obtained by the alkaline lysis method (2). The DNA sequence of a region of *pCIZ1* DNA sequence described in this report was determined.

**Computer analysis of DNA and protein sequences.** Analyses of DNA and protein sequences were performed by using the PC/Genome program package (version 6.8; Intelligenetics, Inc., Mountain View, Calif.) and the Genetics Computer Group package (version 8; University of Wisconsin, Madison).

**Purification and amino acid sequencing of EntL50A and EntL50B.** The anti-

### Table 1. Synergistic antimicrobial activity of in vitro-synthesized EntL50A and EntL50B

<table>
<thead>
<tr>
<th>Indicator species</th>
<th>Strain</th>
<th>Source (^b)</th>
<th>Activity (AU/ml) (^b)</th>
</tr>
</thead>
</table>
| *Pedicoccus acidilacti*
| 347         | FVM         | 2,400       | 51,200               |
| *Enterococcus faecium*
| T136        | FVM         | 1,200       | 19,200               |
| *Lactococcus lactis* subsp. *cremoris*
| CNRZ 177   | INRA        | 4,800       | 25,600               |
| *Lactobacillus sake*  | 148         | FVM         | 600         | 51,200               |

\(^a\) For synergism assays, samples of separately in vitro-synthesized EntL50A and EntL50B were mixed in a 1:1 ratio (EntL50AB). AU, antimicrobial units.

\(^b\) FVM, Facultad de Veterinaria (Madrid, Spain); INRA, Station de Recherches Latières (Jouy en Josas, France).
no obvious sequence homology to any known protein. Further downstream of entL50B is situated an insertion element of the IS3 family termed IS1514 (Fig. 1). The complete sequence of the IS element (1,365 bp) includes terminal inverted repeats of 39 bp and two overlapping ORFs (orfC and orfD) consisting of 288 and 927 bp, respectively (results not shown).

**Purification and amino acid sequencing of EntL50A and EntL50B.** By using a purification procedure which includes ammonium sulfate precipitation and cation-exchange, hydrophobic-interaction, and reverse-phase liquid chromatographies, two purified fractions containing antimicrobial activity were obtained from a 1-l culture of *E. faecium* L50 (results not shown). The procedure was essentially the same as the one originally used by Cintas et al. (6) to purify EntL50, but the last step (reverse-phase liquid chromatography) was rerun several times in an attempt to separate the two peptides. The fractions were subjected to Edman degradation, but since the N termini of both peptides turned out to be blocked, no sequence information was obtained. From the DNA sequence data, it was deduced that the sequences of EntL50A and EntL50B contained two methionine residues. Therefore, samples from the purified fractions were cleaved by CNBr. Subsequent sequencing revealed that one of them was pure and contained enterolactone (EntL50), while the other contained a mixture of enterocin L50A and enterocin L50B. Furthermore, the results of the Edman degradation after CNBr cleavage gave the N-terminal amino acid sequence GAIAKLV, which demonstrates that the deduced gene products of entL50A and entL50B have been
Coexpression of entL50A and entL50B in E. coli. A PCR fragment containing entL50A and entL50B was cloned into the NdeI and HindIII sites of the prokaryotic expression vector pRSETB (Invitrogen Corp.), under control of the strong bacteriophage T7 promoter (48). The recombinant plasmid (pRSETB-entL50AB) could not be established in E. coli JM109 (DE3), probably because small amounts of toxic bacteriocins were produced prior to induction. Even in the absence of IPTG, there is some expression of T7 RNA polymerase from lacUV5 promoter in λDE3 lysogens such as strain JM109 (DE3). For this reason, we selected another bacterial host [E. coli BL21 (DE3)(pLysS)] which contains a compatible plasmid (pLysS) that provides a small amount of T7 lysozyme. This bifunctional protein is a natural inhibitor of T7 RNA polymerase (47). Consequently, background expression can almost be abolished in λDE3 lysogens harboring the pLysS plasmid. As expected, it was possible to establish the recombinant plasmid in the new host, indicating that small amounts of EntL50A and/or EntL50B produced in the cytoplasm are toxic to E. coli. Coexpression of entL50A and entL50B was induced by IPTG at 37°C. Within 60 min after induction, the optical density of the culture producing the bacteriocins dropped from 0.36 to 0.05, whereas the noninduced culture continued to grow normally. Cell-free supernatants of induced and noninduced cultures were assayed at regular intervals by a microtiter plate assay. Antimicrobial activity was first detected after 30 min and reached a maximum after approximately 1 h, in accordance with the observed rate of lysis of the E. coli host cells. No activity was detected in the cell-free supernatants of the noninduced culture.

In vitro transcription/translation of entL50A and entL50B. To investigate whether EntL50A and EntL50B have any antimicrobial activity by themselves, we cloned their structural genes into different pRSETB vectors, resulting in the constructs pRSETB-entL50A and pRSETB-entL50B. Due to the difficulties encountered when the two peptides are expressed together in vivo, we decided to prepare the material needed for further analyses by in vitro transcription/translation. By using the constructs pRSETB-entL50A, pRSETB-entL50B, and pRSETB-entL50AB in coupled in vitro transcription/translation reactions, the two bacteriocins were synthesized separately or together. The in vitro transcription/translation product containing both peptides exhibited strong antimicrobial activity against the indicator strain (P. acidilactici 347), as demonstrated by the sharp 14-mm-diameter inhibition zone observed in the spot-on-agar test (Fig. 4). Interestingly, in vitro-synthesized EntL50A and EntL50B also displayed antimicrobial activity by themselves (Fig. 4). The inhibition zone produced by the sample containing EntL50A was 13 mm in diameter, whereas the zone produced by the corresponding EntL50B sample was only 9 mm in diameter. In both cases, the inhibition zones were less sharp than the larger zone produced by both peptides. These results suggest that the two peptides together possess a greater antimicrobial effect than that exerted by the most active peptide acting alone (EntL50A). To confirm this synergistic effect, we employed a microtiter plate assay to determine the number of antimicrobial units in samples containing either EntL50A, EntL50B, or a mixture of both peptides (see Materials and Methods). The results presented in Table 1 conclusively show that when the two peptides are mixed in about a 1:1 ratio, their antimicrobial effect is much greater than the additive effect of the two bacteriocins acting independently. Furthermore, the degree of synergism varies considerably (4 to 73 times) depending upon which species of indicator bacterium is used. Since the same amount of plasmids (pRSETB-entL50A and pRSETB-entL50B) was added to each in vitro transcription/translation reaction mix, and since the translation products are very small, it is likely that the bacteriocins were synthesized at similar rates. If this is the case, EntL50A is 4 to 12 times more effective than EntL50B in killing the indicator bacteria.

DISCUSSION

The genetic and biochemical data presented in this report show that the antimicrobial activity previously assigned to enterocin L50 arises from two closely related, small, unmodified, cationic, and hydrophobic peptides, termed enterocin L50A and L50B.
and enterocin L50B (Fig. 3). At first glance, the enterocins seem to belong to a large group of antimicrobial compounds called class II bacteriocins. Class II bacteriocins are defined as small, cationic, hydrophobic, and heat-stable modified peptides with antimicrobial activity (31). This class includes bacteriocins consisting of a single polypeptide chain, as well as bacteriocins whose activity depends on the complementary action of two peptides. In some two-peptide systems, e.g., lactococcin G (39) and lactococcin M (53), both peptides are needed for activity. In others, e.g., plantaricin S (29), lactacin F (1), and thermodphilin 13 (36), one or both peptides have some activity on their own but the combined action of both peptides enhances the activity much more than the additive effect of the peptides acting independently. EntL50A and EntL50B seem to have more in common with two-peptide bacteriocins of the last category. However, some characteristics of enterocin L50 peptides clearly distinguish them from class II bacteriocins. (i) The enterocins are secreted without a leader sequence or signal peptide. (ii) Apparently, the structural genes of the enterocins are not cotranscribed with a gene encoding an immunity protein. The immunity proteins of class II bacteriocins, whose genes are located on the same operon as the structural genes, specifically protect the producer bacteria against self-toxicity. (iii) The organization of the enterocin L50 locus is different from class II bacteriocin gene clusters, which in addition to the bacteriocin gene(s) include genes encoding proteins involved in immunity, processing, secretion, and regulation. (iv) The enterocins inhibit growth of a wide range of gram-positive bacteria, whereas most class II bacteriocins have a narrow antimicrobial spectrum. (v) Unlike the enterocins, class II bacteriocins are synthesized as inactive precursors (28, 38), presumably to protect the producer cells against the toxin while it is still in the cytoplasm.

All features considered, the enterocin L50 system seems to have more in common with members of a small group of antimicrobial and/or hemolytic peptides secreted by staphylococci than with class II bacteriocins. These peptides, the δ-lysin, three peptides with hemolytic activity (SLUSH A to C), and three peptides termed antigonococcal substances (AGS 1 to 3), are produced by *Staphylococcus aureus* (17, 33) *Staphylococcus lugdunensis* (15), and *Staphylococcus haemolyticus* (57), respectively. They are ribosomally synthesized, unmodified, secreted without leader sequences or signal peptides, and do not seem to be cotranscribed with an immunity protein gene (15, 17, 33). The antigonococcal substances have not been characterized at the genetic level, but their striking similarity to the SLUSH peptides (Fig. 3) strongly suggests that the characteristics listed above also apply to them. The δ-lysin, which is lytic to a wide range of eukaryotic cells, is a small peptide of only 26 amino acids which ruptures cells by forming pores selective for cations in their plasma membrane (17, 33). As far as we know, the δ-lysin has not been shown to possess antimicrobial activity. The SLUSH and AGS systems both consist of three closely related peptides (Fig. 3), which exhibit hemolytic activities on erythrocytes from various species (15, 57). In addition, the AGS peptides have antimicrobial activity, showing a broad antigonococcal spectrum and a narrow antibacterial spectrum (57). Preliminary data suggest that the SLUSH peptides also have the ability to kill bacteria. It has been shown that crude extracts from hemolytic, but nonhemolytic, *S. lugdunensis* strains inhibit growth of other staphylococci (15). However, since bacteria often produce multiple toxins, it is uncertain whether this antimicrobial activity should be ascribed to the SLUSH peptides. Considering the similar features of the enterocins and the staphylococcal toxins, we wondered if the enterocins EntL50A and EntL50B possess hemolytic activity as well. This hypothesis was tested by growing *E. faecium* L50 on calf, horse, porcine, and human blood agar plates. Furthermore, in vitro-synthesized EntL50A and EntL50B were spotted on corresponding blood agar plates, either separately or in combination. Both assays were negative, indicating that the enterocins have no hemolytic activity (data not shown). Evidence of a relationship between the enterocins and the SLUSH and AGS peptides was obtained by comparing their primary structures. This comparison revealed a low but significant homology between the enterocins and the staphylococcal peptides (Fig. 3). Together, amino acid sequence homology and other unique characteristics shared by the enterocins and the staphylococcal peptides strongly indicate that they constitute a separate, hitherto-unrecognized, family of peptide toxins.

Ruminicent of the SLUSH peptides, the genes encoding the enterocins are situated next to each other, presumably on a single transcription unit. The simplicity of this locus is striking compared to the organization of the gene clusters involved in production of class II bacteriocins. For instance, none of the ORFs neighboring the structural genes of the enterocins seem to be involved in their secretion. The secretion machinery of the staphylococcal hemolysins has not been identified, but it does not seem to be located close to the structural genes. Many larger bacterial proteins are known to be secreted without an N-terminal leader sequence or signal peptide. These include *E. coli* hemolysin (10, 16, 21), *Pasteurella haemolytica* leukotoxin (46), *Erwinia chrysanthemi* and *Serratia marcescens* proteases (11, 56), *Bordetella pertussis* cyclosin (19), and the virulence plasmid-encoded proteins termed Yops (*Versinia* outer membrane proteins) (35, 37). It has been established for most of them that secretion is mediated by specific ABC transporters (24), rather than by the secretion apparatus encoded by the GSP genes (4), and that a stretch of about 50 amino acids at their C termini targets them for export (35, 45). Even though no genes encoding a possible transport protein have been identified on the sequenced 3.5-kb fragment, we speculate that EntL50A and EntL50B are secreted by a dedicated ABC transporter, as demonstrated for the larger proteins discussed above.

Ribosomally synthesized prokaryotic proteins initially contain an N-terminal formylmethionine, which is usually removed together with the leader sequence or signal peptide in extracellular proteins. Both EntL50A and EntL50B are blocked for direct N-terminal amino acid sequencing by Edman degradation, indicating that the N-terminal methionine is modified or that the formylmethionine is retained. We favor the last possibility since it has been reported that about 90% of the δ-lysin molecules contain formylmethionine at their N termini after secretion (17). Microcin C7, another ribosomally synthesized antimicrobial peptide lacking a leader sequence or signal peptide, is also secreted without removal of the N-formyl group (20). In addition, it is known that this kind of modification will block Edman degradation.

For all the four species tested, the levels of antimicrobial units of EntL50A are much higher (4 to 12 times) than the corresponding values of EntL50B. There could be two reasons for this: (i) the in vitro transcription/translation reactions produce more EntL50A than EntL50B or (ii) EntL50A is more active against the bacteria tested than EntL50B. Since in vitro synthesis of the peptides was carried out in parallel with the same amount of plasmids, we consider it unlikely that quantitative differences alone can account for the much higher levels of antimicrobial units determined for EntL50A. If future investigations confirm that EntL50A is more active than EntL50B, domain swapping and site-directed mutagenesis could be used to pinpoint which amino acid residues make EntL50A the most active antimicrobial compound. Most likely,
the C-terminal part of the molecule will be found to play a crucial role.

Elucidation of the mechanisms involved in the secretion, immunity, and mode of action of enterocins L50A and L50B, as well as identification of structural motifs required for their broad antimicrobial activity, may be helpful in the development of new antimicrobial compounds to be used in either the pharmaceutical or the food industry.

ACKNOWLEDGMENTS

This work was partially supported by the Commission of the European Communities (project contract Bio CT-96-5051). Luis M. Cintas was a recipient of a Postdoctoral Research Training grant from the Commission of the European Communities.

We are indebted to Knut Sletten (University of Oslo, Oslo, Norway) for amino acid sequencing.

REFERENCES


