

Specific Degradation of the Mucus Adhesion-Promoting Protein (MapA) of *Lactobacillus reuteri* to an Antimicrobial Peptide[∇]

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The intestinal flora of mammals contains lactic acid bacteria (LAB) that may provide positive health effects for the host. Such bacteria are referred to as probiotic bacteria. From a pig, we have isolated a *Lactobacillus reuteri* strain that produces an antimicrobial peptide (AMP). The peptide was purified and characterized, and it was unequivocally shown that the AMP was a well-defined degradation product obtained from the mucus adhesion-promoting protein (MapA); it was therefore termed AP48-MapA. This finding demonstrates how large proteins might inherit unexpected pleiotropic functions by conferring antimicrobial capacities on the producer. The MapA/AP48-MapA system is the first example where a large protein of an intestinal LAB is shown to give rise to such an AMP. It is also of particular interest that the protein that provides this AMP is associated with the binding of the bacterium producing it to the surface/lining of the gut. This finding gives us new perspective on how some probiotic bacteria may successfully compete in this environment and thereby contribute to a healthy microbiota.

Mammals have a microbiota in their digestive tract that contains lactic acid bacteria (LAB). It has been increasingly evident that some of these lactic acid bacteria produce antimicrobial peptides that may contribute to the positive effect on their host. Such bacteria are often referred to as probiotics, and one of their important beneficial effects is their ability to produce antimicrobial compounds that prevent or interfere with the growth of pathogenic bacteria in the host.

It is known that the fecal microflora of pigs/piglets is large and diverse and develops rapidly after birth. *Lactobacillus reuteri* is among the very first lactic acid bacteria that colonize the intestine of new-born piglets, and their numbers gradually increase until they become the most dominant LAB in pigs (5, 17, 28). Other lactobacilli that are also part of the gut microbiota of pigs include *L. amylovorus*, *L. acidophilus*, *L. salivarius*, and *L. casei* (4, 8). Probiotic isolates have been identified within all these species, and many of them are today used as food/feed supplements to support good health (4, 11, 27). An important part of the antimicrobial arsenal produced by lactic acid bacteria (LAB) is a group of peptides called bacteriocins, which are ribosomally synthesized antibiotic-like peptides (antimicrobial peptides [AMPs]) (3, 7, 19). The bacteriocins constitute a wide range of structurally different peptides that are divided into different classes and subclasses. Some are modified (the lantibiotics, or class I), while others are basically unmodified (class II) (3, 6, 19).

Most bacteriocins are derived from prepeptides, each containing a short leader sequence (14 to 30 amino acids [aa]) which is cleaved off during the secretion of the mature peptide (19). In recent years, a new group of AMPs have been recog-

nized (18); these are different from regular bacteriocins in that they are derived from larger proteins through specific degradations, leading to a defined peptide possessing antimicrobial activity. Such antimicrobial peptides have been known for a long time in mammalian systems. For instance, lactoferrin, a protein in milk, is readily degraded to a specific antimicrobial peptide through heat, acid treatment, or pepsin digestion (14, 24, 26). Defined histone fragments with antimicrobial properties have been isolated from different eukaryotic species (1, 2, 15, 21, 23), and a few antimicrobial peptides derived from larger proteins have been isolated in bacteria, including *Helicobacter pylori* (22), propionic acid bacteria (9, 10), and *Clostridium beijerinckii* (13). Such antimicrobial peptides are most likely formed by proteolytic degradation during cell proliferation or death.

Isolation of antimicrobial-producing LAB from piglets. Fecal samples were collected from 4 different litters of piglets (2 to 4 weeks old), and LAB were isolated by plating serial dilutions of samples onto MRS agar plates which were incubated anaerobically at 37°C. Altogether, 37 LAB were isolated. They were dominated by *L. reuteri* (13 isolates), but *Lactobacillus kitasatonis*, *L. amylovorus*, *L. salivarius*, *Lactobacillus pontis*, *Lactobacillus mucosae*, and *Lactobacillus vaginalis* were found, in addition to *Streptococcus bovis* and enterococci. The typing was performed by 16S RNA DNA sequencing. The DNA sequence was based on 1,182 nucleotides, and BLAST analysis showed 99% identity only to *L. reuteri* or some uncultured bacteria obtained from fecal pig or human gut microflora.

The antimicrobial activity was tested by serial 2-fold dilution of samples and growth inhibition of an indicator organism in microtiter plates as previously described (12). Several of the isolates were found to produce antibacterial activity, and among them, the fecal isolate *Lactobacillus reuteri* LA92 displayed a fairly broad antimicrobial profile, including inhibition of selected members of the pathogenic bacteria *Bacillus cereus*, *Staphylococcus aureus*, and *Listeria monocytogenes*, as well as

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TABLE 1. Overview of the purification procedure of the antimicrobial peptide from *L. reuteri* LA92

Fraction	Volume (ml)	Antimicrobial activity (BU/ml) ^a	Total activity (BU) ^b	Yield (%)
Supernatant	600	20	12,000	
Precipitation with (NH ₄) ₂ SO ₄	60	320	19,200	100
Ion exchange chromatography	10	1,280	12,800	67
Reversed-phase chromatography (Resource column)	1	500	500	2.6
Reversed-phase chromatography (C ₈ column)	0.5	4,800	2,400	12.5
Reversed-phase chromatography (C ₈ -s column)	0.5	400	200	1.04

^a One bactericidal unit (BU) is the minimum amount of the antimicrobial peptide that inhibits the growth of the indicator by 50%. The indicator organism used in the bacteriocin assay was *L. sakei* LMG2313.

^b Total activity is the product of the volume of the active fraction (ml) and the activity of that fraction (BU/ml).

some lactic acid bacteria but not *Lactobacillus plantarum*. Antimicrobial activity was not detected against *Escherichia coli* or a *Salmonella* isolate. No activity was lost upon heat treatment (95°C for 10 min), but the activity was sensitive to proteinase K treatment, together suggesting that the antimicrobial compound is heat stable and proteinaceous, characteristics which are typical of most bacteriocins. However, we will not describe this antimicrobial peptide as a bacteriocin since it seems to lack many of the other characteristics typical for bacteriocins (19); we will instead refer it to as an antimicrobial peptide.

Purification of the antimicrobial compound. The compound was secreted into the growth medium, from which the activity was purified according to well-established protocols (20, 25). Briefly, the cell-free supernatant from 600 ml of an overnight culture of *L. reuteri* LA92 was precipitated with 240 g of ammonium sulfate. The protein precipitate was pelleted by centrifugation at 13,000 × *g* for 20 min at 4°C and dissolved in 60 ml distilled water. This fraction was applied to an ion exchange chromatograph with an SP-XL column. The antimicrobial activity that was bound to the column was washed once with 0.1 M NaCl (15 ml) before being eluted from the column by 1 M NaCl (10 ml). The fractions containing antimicrobial activity were pooled and further subjected to reversed-phase chromatography (RPC), which consisted of one run on a Resource RPC 1-ml column and two runs on a Sephacil peptide C₈ column. An isopropanol gradient from 1 to 100% isopropanol in 0.1% trifluoroacetic acid was used to elute the antimicrobial activity. Table 1 summarizes the yields of antimicrobial activity at the different steps of purification. The antimicrobial activity in two fractions (nr 24 and nr 25) (Table 2) eluted at 66.7% isopropanol gave a sharp absorbance (280 nm) peak with some tailing. The two fractions with antimicrobial activity, as well as

the two flanking fractions, were analyzed by mass spectrometry (MS). A monoisotopic mass of 5,305.6 Da was identified as the major component in the fraction (nr 24), with 80% of the antimicrobial activity (Fig. 1). The complete mass analyses of all four fractions are presented in Table 2. Several mass peaks were identified, but the 5,306-Da peak was recognized only in the two fractions (nr 24 and nr 25) with antimicrobial activity.

Fraction nr 24, which represented the majority of the activity in the final step, was subjected to peptide sequence analysis by automated Edman degradation as previously described (12). The sequence of the first 19 amino acids was identified (LEG TYPYSYRKNNKLTGF). In addition to this N-terminal sequencing, the compound was also fragmented by tandem mass spectrometry, and another sequence of 19 amino acids was identified (KNNKLTGFEVDLGKAVAKK); this sequence overlapped with the 8 aa residues from Edman degradation. Altogether, a stretch of 30 amino acids of the purified AMP was identified, as shown in Fig. 2.

A BLAST search showed that this 30-aa peptide sequence was identical with an internal sequence (from residue 46 to residue 75) of the 263-aa protein called MapA (mucus adhesion-promoting protein) encoded in the *L. reuteri* genome (E. Satoh, R. J. Leer, M. Rojas, P. L. Conway, F. J. Tielen, and P. H. Pouwels, unpublished data; GenBank sequence accession number AJ293860) (16). The molecular mass of the purified antimicrobial peptide (5,305.6 Da), now named AP48-MapA, matched with the theoretical mass of an internal sequence of Map A, from residue 46 to residue 93, that gives rise to a sequence of 48 aa with a molecular mass of 5,308.1 Da. MapA contains an N-terminal *sec* leader (residues 1 to 27) which is probably cleaved upon export of the mature protein, which in turn is processed further from both ends to give rise to AP48-MapA (Fig. 2). The second peak (5,065 Da) in the MS analyses also matched with a truncated peptide sequence (residue 46 to residue 90), of which the theoretical mass was 5,066.8 Da. Another two mass peaks, of 3,678 Da and 3,877 Da, corresponded well with truncated sequences of MapA, one fragment containing residues 46 to 78, with a theoretical mass of 3,679 Da, and the other containing residues 46 to 80, with a theoretical mass of 3,878.5 Da. However, fractions (nr 23 and nr 26) containing these smaller peptide fragments did not show any antimicrobial activity.

In order to provide further evidence that the antimicrobial activity was embedded in the purified peptide of 48 residues, a DNA fragment encoding the peptide was cloned in *E. coli* by the pET expression system (pET-22b+; Novagen). Positive

TABLE 2. MS analysis and antimicrobial activities of some selected fractions from the last step of purification

Fraction	MS peak(s) (Da)	Antimicrobial activity (BU/mg) ^b
Nr 23	3,877	0
Nr 24	5,065, 5,306 ^a	22,200
Nr 25	3,678, 5,063, 5,306	5,000
Nr 26	3,678, 5,063	0

^a Major peak; see Fig. 1.

^b One BU (bactericidal unit) is the minimum amount of the antimicrobial peptide that inhibits growth of the indicator by 50%. The protein concentration is based on a value of 1 mg/ml at an optical density at 280 nm of 1.88; the protein concentrations in fractions 24 and 25 were 18 µg/ml and 20 µg/ml, respectively.

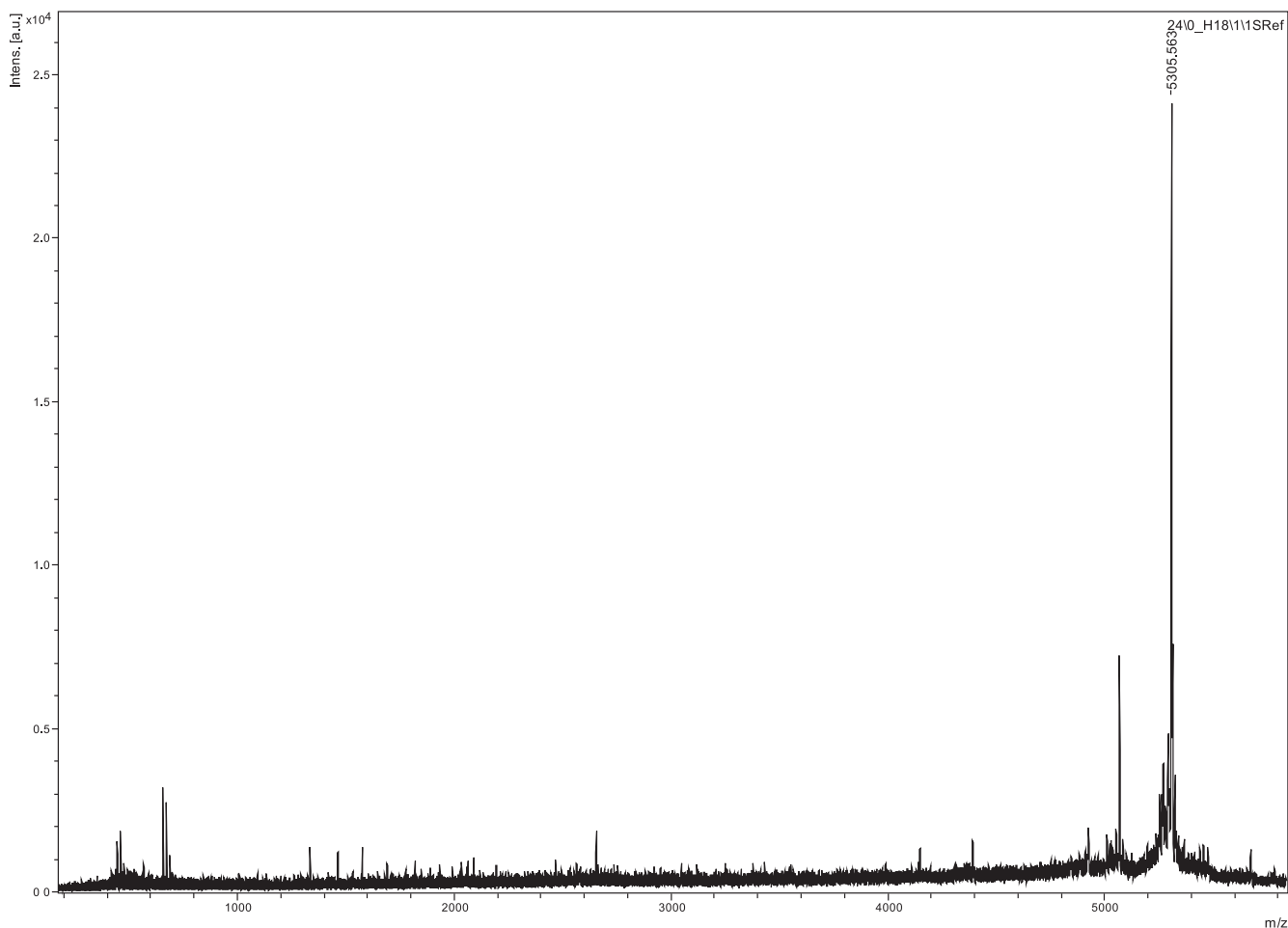


FIG. 1. Mass spectrometry results for the fraction (nr 24) with the highest antimicrobial activity. Intens., intensity; a.u., arbitrary units.

clones were isolated, but upon induction of the expression of the inserted gene, the host (*E. coli*) did not survive, and more than 99% of the cells died. The surviving cells did not produce any antimicrobial activity.

The present work has identified an antimicrobial activity produced by *L. reuteri* isolated from fecal samples of weanling

piglets. The peptide appears to be produced in a specific degradation process of the mucus adhesion-promoting protein (MapA) of *L. reuteri* (E. Satoh et al., unpublished data; GenBank sequence accession number AJ293860) (16). It has occasionally been shown that both bacteria and eukaryotes produce antimicrobial peptides by specific proteolytic degradation of larger proteins. In bacteria, this has been seen in *H. pylori* when the ribosomal protein L1 is degraded (22). In two different propionic acid bacteria, it has been shown that larger proteins of unknown function are degraded to form antimicrobial peptides. Likewise, histones in eukaryotes have been shown to be degraded to distinct peptides with strong antimicrobial activity (16–20).

L. reuteri is part of the intestinal LAB of pigs, and it is likely that it contributes positively to the gastrointestinal health of the animal. It is certainly tempting to speculate that the AMP derived from MapA is one of the factors that protect the carrier against invading pathogenic bacteria. Since the major function of MapA is probably to attach the bacterium to the lining of the gut, it is also likely that during propagation, bacteria are released into the lumen and degradation of the MapA peptide will take place. Also, degradation of MapA probably occurs among the bacteria when they are attached to the mucus layer.

A
 MKFWMKALLTIVALTVGTPAGITSVSAASSAVNSELVHKGELTIG**LECTYSPYSYRKNKLTGFVDL**
GKAVAKMGLKANFVPTKWDSLIALGLSGKFDVVMNITQTPERAKQYNFSTPYIKSRFALIVPTDSN
 IKSLKNIKGKKI IAGTGTNNANVVKKYGNLTPNGDFASSLDMIKQGRAAGTINSREAWYAYSKKNST
 KGLKMI DVSSQDPARI SALFNKRDTAIQSSYNKALKELQQDGTVKKLSKYPGADITE

B
 46-**LECTYSPYSYRKNKLTGFVDL**GKAVAKMGLKANFVPTKWDSL-93; MW: 5308.1
 46-**LECTYSPYSYRKNKLTGFVDL**GKAVAKMGLKANFVPTKWDSL-90; MW: 5066.8
 46-**LECTYSPYSYRKNKLTGFVDL**GKAVAKMGLKA-80; MW: 3878.5
 46-**LECTYSPYSYRKNKLTGFVDL**GKAVAKMGL-78; MW: 3679.3

FIG. 2. (A) Amino acid sequence of the MapA protein. (B) Truncated peptides of MapA, whose molecular weights (MW) matched well with the molecular masses of the purified peptides determined by mass spectrometry. MapA (accession no. AJ293860) is a mucus adhesion protein (263 aa) containing a *sec* leader sequence (underlined). Note that all truncated peptides have a common N terminus, but each has a different C terminus. Bold letters indicate the sequence determined by chemical sequencing of purified peptides.

Several degradation products from MapA were identified, but only one dominating peptide seems to be responsible for the antimicrobial activity, namely, AP48-MapA, which is a small (48-aa), heat-stable peptide with a high pI (9.63), physicochemical properties that are typical of bacteriocins. The processing/degradation required to produce AP48-MapA is not known, but all the peptides identified from the various fractions of the last reversed-phase column appeared to have a common N-terminal processing site at aa residue 48, while the C-terminal degradation site varied according to the MS data. Since the production of AP48-MapA was found in the supernatant of a monoculture, it seems likely that the bacterium itself can carry out this specific processing in the gut without depending on other enzymatic activity.

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