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Analysis of the Pediocin AcH Gene Cluster from Plasmid pSMB74 and Its Expression in a Pediocin-Negative *Pediococcus acidilactici* Strain†

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The 3,500-bp *pap* operon in the 8,877-bp plasmid pSMB74 contains a cluster of four genes, *papABCD*, of which *papA* encodes prepediocin (A. M. Motlagh, M. Bukhtiyarova, and B. Ray, Lett. Appl. Microbiol. 18:305–312, 1994). The cluster without the promoter was cloned in the shuttle vector pHPS9. An *Escherichia coli* strain and a pediocin-sensitive *Pediococcus acidilactici* strain transformed with the recombinant plasmid, pMBR1.0, produced pediocin AcH. Deletion analysis by introducing mutations in the four genes in pMBR1.0 revealed that only *papA* and *papD* were required for pediocin AcH production and that the gene product of *papD* has both translocation and processing functions. In the transformed minicells of *E. coli* χ925 the proteins of the *pap* cluster were synthesized, indicating no polar effect due to deletion.

Bacteriocins of gram-positive bacteria first are translated as prepeptides and then undergo maturation, processing, and transmembrane translocation before the active molecules are excreted in the environment (2). Many of the posttranslation steps are enzyme mediated. Generally, the genes encoding these enzymes and the immunity protein are located in a gene cluster along with the structural gene of a prepeptide (3, 4, 6, 10–14, 21, 23). The structural gene encoding pediocin AcH (Pap^+) in *Pediococcus acidilactici* LB42-923 is located in a cluster of four genes (the *pap* cluster) in an 8,877-bp plasmid, pSMB74 (13, 14, 17). The four genes *papA*, *papB*, *papC*, and *papD* encode proteins of 62, 112, 174, and 724 amino acid residues, respectively. The 62-amino-acid residue is prepediocin, from which an 18-amino-acid residue from the N terminus is removed by proteolytic cleavage. The remaining 44-amino-acid residue is pediocin AcH, which probably has two disulfide bridges between four cysteine residues (7, 12–14). Indirect evidence has suggested that pSMB74 also encodes genes for proteins necessary for immunity, processing, and translocation phenotypes (8, 15, 16, 18, 19). The role of proteins encoded by *papBCD* genes in these functions was studied and is reported here.

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used and their sources are listed in Table 1. *P. acidilactici* strains were grown in tryptone-glucose-yeast extract (TGE) (1) broth or agar plates with or without chloramphenicol (Cm) (5 μg/ml) and erythromycin (Em) (10 μg/ml), and *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or agar plates with or without Cm (5 μg/ml) or Em (100 μg/ml) at 37°C. *Lactobacillus plantarum* NCDO 955 was used as a sensitive indicator to detect the presence of pediocin AcH (1, 24).

DNA manipulation. Plasmid DNA from *P. acidilactici* and *E. coli* was isolated by previously described methods (18, 20) and by the Wizard Minipreps procedure (Promega Corporation, Madison, Wis.). Restriction enzymes, T4 DNA ligase, and other DNA-modifying enzymes were used as recommended by the manufacturers. The cloning procedures, agarose gel electrophoresis, and transformation of *E. coli* (by $CaCl_2$ procedure) and *P. acidilactici* (by electroporation) were performed according to established procedures (9, 20). DNA fragments from agarose gel were purified by the Magic PCR Preps procedure (Promega Corporation).

Cloning of *pap* cluster genes in a shuttle vector and expression of pediocin AcH. Plasmid pSMB74 was digested with restriction enzyme *Bsp* 501, and the 3,478-bp fragment that contained the four genes and the terminator but not the promoter was ligated to *Sma*I-digested pHPS9 (5). The recombinant plasmid, pMBR1.0, was transformed in *E. coli* JM109, and the transformants were selected on LB agar plates containing Em and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). A transformant was grown in LB broth, transferred by pour plate technique in LB agar, and incubated for 16 h to form colonies. The plates were overlaid with TGE soft agar seeded with *L. plantarum* NCDO 955, incubated for an additional 24 h, and examined for the zone of growth inhibition around the colonies (15, 18, 19). *P. acidilactici* LB42 cells were electrotransformed with vectors pHPS9 and pMBR1.0 (1 μg/10^8 cells), and transformants were selected on TGE agar plates containing Em and Cm. The ability of the transformants (with pMBR1.0) to produce pediocin AcH was detected on TGE agar plates against a lawn of *L. plantarum* NCDO 955 by the methods described above for *E. coli.*

Construction of plasmids with mutation in genes in the *pap* cluster. Frameshift mutation was used to introduce deletions in the four genes in pMBR1.0 (20). The plasmid was digested with one or two suitable restriction enzymes, the fragments were separated on agarose gel, and the fragments of interest were eluted and blunt ended by Klenow enzyme with a deoxynucleoside triphosphate mixture. Following transformation of a modified plasmid in *E. coli* JM109, the accuracy of deletion of a gene was determined by the absence of the original restriction site(s). For deletions in genes in pMBR1.0 and to construct the corresponding plasmid derivatives, the following enzymes were used: *Bal*I-*Esp*I in *papA* for pMBR1.1, *Esp*I-*Bsm*I in *papB* for pMBR1.2, *Sca*I in *papC* for pMBR1.3, *Bgl*I in *papD* for pMBR1.4, *Esp*I-*Sca*I in *papB* and *papC* simultaneously for pMBR1.23, and *Hin*dIII in *papBCD* simul-

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TABLE 1. Bacterial strains and plasmids used in this study and their sources

Strain or plasmid	Characteristic(s)	Reference(s) or source
P. acidilactici LB42-923	Pap$^+$ Pai^r, pSMB74	19
P. acidilactici LB42	Pap$^-$ Pai^s, plasmidless, Cm^s Em^s	19
P. acidilactici LB42-1.0	Pap$^+$ Pai^r Cm^r Em^r, pMBR1.0	This study
L. plantarum NCDO 955	Pap$^-$ Pai^s, indicator	15, 24
E. coli JM109	Host	Stock
E. coli χ925	MinA MinB	22
pSMB74	Source of *pap* cluster	13, 14, 17
pHPS9	*E. coli-Bacillus subtilis* vector, Cm^r Em^r	5
pMBR1.0	*pap* cluster in pHPS9	This study
pMBR1.1	pMBR1.0 with *papA* mutation	This study
pMBR1.2	pMBR1.0 with *papB* mutation	This study
pMBR1.3	pMBR1.0 with *papC* mutation	This study
pMBR1.4	pMBR1.0 with *papD* mutation	This study
pMBR1.23	pMBR1.0 with *papBC* mutation	This study
pMBR1.234	pMBR1.0 with *papBCD* mutation	This study

taneously for pMBR1.234. All six recombinant plasmids of pMBR1.0 were transformed in *E. coli*, and the ability of the transformants to produce pediocin AcH was determined by the method described earlier. Electrotransformation of *P. acidilactici* LB42 with pMBR1.2 and pMBR1.3 and assays of the ability of the transformants to produce pediocin AcH were also conducted as before.

Detection of proteins of *pap* genes in minicells of *E. coli*. Following transformation of *E. coli* χ925 (MinA MinB producer) with pMBR1.0 and its deletion derivatives, minicells were prepared (22). To label the proteins produced, minicells were incubated in LB broth containing L-[^{35}S]methionine. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiography (20, 24).

Expression of *pap* gene cluster phenotype. Cells of both *E. coli* and a pediocin-sensitive *P. acidilactici* strain transformed with the recombinant plasmid pMBR1.0 were capable of producing pediocin AcH (Fig. 1). An absence of growth inhibition zone around colonies treated with chymotrypsin, before an overlay with indicator, confirmed the production of pediocin AcH by these transformants (data not shown). Judging from colony and zone sizes, production of pediocin AcH seemed to be less in *E. coli* than in *P. acidilactici* transformants. The phenotypes of the *pap* gene cluster are also expressed without the promoter in a shuttle vector. Marugg et al. (12) cloned the four genes with the promoter in a shuttle vector and reported production of pediocin PA-1 (which essentially is the same as pediocin AcH) by a transformed *E. coli* strain only. The transformation frequency of pMBR1.0 was higher in *E. coli* than in *P. acidilactici*, and in both strains, especially in *P. acidilactici* transformants, the Pap$^+$ phenotype was unstable. Plasmid analysis of Pap$^-$ Cm^r Em^r variants of the Pap$^+$ Cm^r Em^r transformants of *P. acidilactici* showed the presence of a smaller plasmid, close to the size of the vector (data not included). The cloned fragment from pSMB74 may be unstable in pHSP9, especially in *P. acidilactici*. Analysis of the cell wall and cytoplasmic fractions of the transformed *E. coli* cell lysates showed no pediocin AcH activity (data not shown). These cells

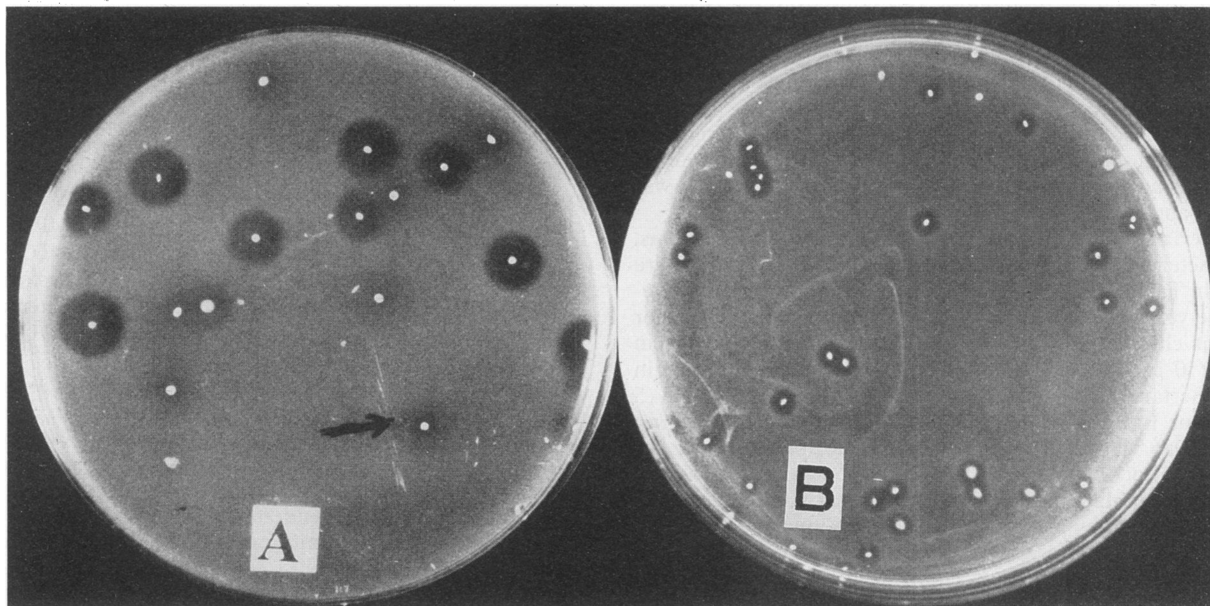

FIG. 1. (A) *P. acidilactici* LB42-1.0 carrying pMBR1.0 following electroporation shows a large clear zone of growth inhibition of indicator *L. plantarum* NCDO 955 around the colonies. However, the Pap$^+$ phenotype was unstable in the transformants, as indicated by the absence of a clear zone around some colonies (arrow; light zone was due to acid). These Pap$^-$ variants are Cm^r Em^r and carry a smaller plasmid about the size of the vector. (B) *E. coli* JM109 carrying pMBR1.0 and producing pediocin AcH, as determined by a small clear zone around the colonies. Some Pap$^-$ variants were also present. Treatment of the agar surface with chymotrypsin prior to an overlay with indicator bacteria eliminated formation of a clear zone around the colonies of both transformant strains.

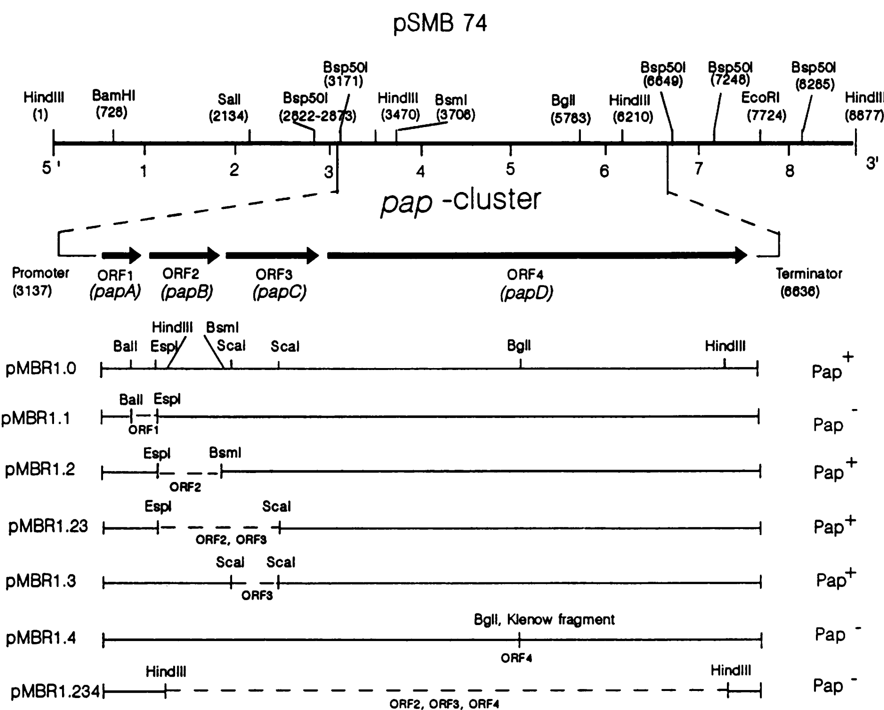

FIG. 2. Pediocin AcH-encoding plasmid pSMB74, *pap* gene cluster, pMBR1.0, and six deletion derivatives of pMBR1.0. In pSMB74, locations of several restriction sites (with nucleotide positions in parentheses) are indicated. The *pap* gene cluster is shown with the promoter at bp 3137 and the terminator at bp 6636 and with the four open reading frames (ORFs) 1, 2, 3, and 4 corresponding to genes *papA*, *-B*, *-C*, and *-D*, respectively. The six deletion derivatives show the specific restriction enzymes used to remove the DNA fragments. The ability or inability to produce pediocin AcH (Pap$^+$ or Pap$^-$, respectively) of the *E. coli* JM109 transformants carrying pMBR1.0 or the deletion derivatives is indicated on the right. The different pediocin AcH-producing transformants produced zones of growth inhibition around colonies of about the same size as those indicated in Fig. 1B. Distances on the restriction map are indicated in increments of one thousand.

appeared to secrete all pediocin AcH in the environment. Passage of bacteriocin molecules through the porous cell wall of gram-positive bacteria is assumed to be a passive process (10). How pediocin AcH molecules passed through the outer membrane of *E. coli* is not known. The passage of bacteriocin molecules through the cell wall and outer membrane may not be automatic.

Deletion analysis of *pap* gene cluster. The ability of *E. coli* strains transformed with deletion derivatives of pMBR1.0 to produce pediocin AcH was studied (Fig. 2). While the transformants carrying recombinant plasmids with deletions in either *papB*, *papC*, or *papBC* produced pediocin AcH, those with deletions in *papA*, *papD*, or *papBCD* failed to do so. Thus, in *E. coli* only *papA* and *papD* are required for pediocin AcH production. Since *papA* encodes prepediocin, the protein encoded by *papD* is capable of both translocation and processing of the molecules. A translocation function of PapD protein has been suggested before (14). Marugg et al. (12) in a related study introduced mutations in *pedA*, *pedB*, and *pedD* but not in *pedC* (genes similar to *papABCD*), which showed *pedB* was not necessary for pediocin PA-1 production and suggested the function of PedD protein was secretion of pediocin. We transformed pediocin-sensitive *P. acidilactici* LB42 with both pMBR1.2 (deletion in *papB*) and pMBR1.3 (deletion in *papC*). Transformants (Cm^r Em^r) for pMBR1.3 were isolated at a frequency of about $10^5/\mu g$ of DNA (same frequency as with the vector), but not one was isolated for pMBR1.2 in three separate trials. However, none of the 50 transformants for pMBR1.3 examined produced pediocin AcH, but they had a plasmid smaller than pMBR1.3 (data not shown). This could be due to instability of the recombinant plasmid in *P. acidilactici*, as explained before. The function of the *papC* gene could not be determined. From the similarities in hydropathy curves between PapC, LcnD protein in the lactococcin A operon, and HylD protein in *E. coli*, it can be inferred that PapC facilitates the secretory function of the PapD protein (21; data not shown). Marugg et al. (12) suggested that the PedC protein is similar to the SpaD protein and is associated with pediocin synthesis. The inability to obtain transformants with pMBR1.2 in pediocin-sensitive *P. acidilactici* is suggestive of the role of the *papB* gene for encoding immunity protein. These transformed cells are capable of producing pediocin AcH but in the absence of immunity protein were killed and failed to produce colonies. Marugg et al. (12) recognized that the immunity gene against pediocin PA-1 is chromosomally linked but because of the location of *pedB* close to *pedA* suggested that PedB was the immunity protein.

Expression of deletion derivatives of *pap* cluster in minicells of *E. coli* χ925. Minicells carrying vector pHPS9 (control) synthesized several proteins (Fig. 3, lane 1). When compared with the control, minicells carrying either pMBR1.0 (lane 3) or pMBR1.1 (lane 2) produced proteins with molecular masses of 11.5 (B), 19.0 (C), and about 80 (D) kDa. The calculated masses of the deduced amino acid sequences of the three proteins encoded by *papBCD* genes, respectively, are 13.0, 19.2, and 81.7 kDa (14). The calculated data agree well with the molecular masses of the three proteins in the gel. The gene product of *papD* was not distinct in the autoradiograph. This could be due to instability of this membrane-bound large protein. Minicells with pMBR1.3 (Fig. 3, lane 5), pMBR1.23

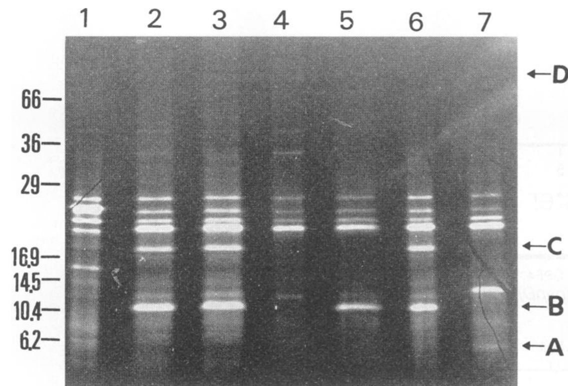

FIG. 3. Autoradiograph of SDS-PAGE showing profile of proteins synthesized by the minicells of *E. coli* χ925, carrying pMBR1.0 or its deletion derivatives, during incubation with L-[^{35}S]methionine. Plasmids carried by the cells were pHPS9 (vector; control) (lane 1), pMBR1.1 (lane 2), pMBR1.0 (lane 3), pMBR1.23 (lane 4), pMBR 1.3 (lane 5), pMBR1.4 (lane 6), and pMBR1.234 (lane 7). Molecular mass in kilodaltons is indicated on the left. Four protein bands (A, B, C, and D) not present in the control but present in cells carrying different plasmids are marked by arrows.

(lane 4), and pMBR1.4 (lane 6) produced proteins B and D, D, and B and C, respectively. These data agreed well with the gene deletion pattern in the plasmids; however, protein D was not clearly visible in lane 5. Minicells with pMBR1.234 (lane 7) had only protein band A of 6.5, kDa, which is about the molecular mass of the *papA* gene product. In addition, lane 7 had a band of 14 kDa. It is most likely the protein encoded by the proximal part of *papB* and distal part of *papD*. Although expected, band A did not appear in lane 6 and a band of 4.6 kDa for pediocin AcH was not present (or not distinct) in lanes 2, 3, 4, and 5. The results showed that deletion of one gene did not have a polar effect on the transcription of gene(s) downstream. We plan to purify the proteins and determine their characteristics.

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