FACTORS INFLUENCING IMMUNITY AND RESISTANCE OF *PEDIOCOCCUS* ACIDILACTICI TO THE BACTERIOCIN, PEDIOCINE AcH

(Faktor-faktor yang mempengaruhi imunitas dan ketahanan *Pediococcus acidilactici* terhadap bakteriosin, pediosin AcH)

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ABSTRAK

Di dalam pediosin Ach yang dihasilkan oleh bakteri *Pediococcus acidilactici*, kedua gen untuk produksi pediosin dan kekebalan terhadap pediosin dikode oleh plasmid pSMB74 pada 8,9 kb. Dengan hilangnya plasmid ini dapat menghilangkan kemampuan bakteri untuk menghasilkan pediosin AcH, tetapi beberapa masih bertahan terhadap pediosin. Bakteri yang bertahan adalah jenis antara yaitu diperoleh pada saat sel yang tidak dapat berproduksi ditumbuhkan pada media yang mengandung pediosin AcH, tetapi hilang ketika sel ditunbuhkan tanpa adanya pediosin AcH.

INTRODUCTION

Bacteriocin production in lactic acid bacterial strains in encoded either by chromosomal genes or on plasmid (Ray 1992a,b,c). The cells or producer starin are immunity to their own bacteriocin due to the presence or specific immunity gene. In several strains in which the bacteriocin production phenotype in plasmid-linked, genetic analysis has shown that the genes both for production of and immunity against a bacteriocin are located close to each other (Hasting and stiles 1991; van Belkum *et al* . 1992). Loss of the bacteriocin plasmid in some strains has produce variants that have lost both the production of on specific immunity against the bacteriocin (Daeschel and Klaenhamer 1985; Ra*y at al.* 1989a; hasting and Stiles, 1991). However, in some other strains, loss of the bacteriocin plasmids has resulted in variants that no longer produced the bacteriocin but retain the immunity (resistance) against the specific bacteriocin. Possible chromosomal linkage of the specific immunity genes in these strains has been suggested (Jimenez-Diaz *et al.* 1993;Schved *et al.* 1993)

Our plasmid transfer and genetic analysis studied for a bacteriocin, pediocin AcH, produced by *Pediococcus acidilactici* strain H have conclusively shown that the 8377 bp plasmid, pSMB74, encodes for both pediocin AcH production (Pap^+) and immunity (Pai^+) against it (Ray et al. 1989b; Johnson et al 1992). However, our initial studies with another three pediocin AcH producingstrain of Ped. Acidilactici, E,F and M, all carrying pSMB74, have resulted in the isolation of non producer variant that are resistance to the pediocin AcH (Kim et al. 1992).

We investigated the reason why loss of pSMB74, that encodes both Pap^+ Pai^+ phenotypes, results in some variants which are sensitive (Pap^s) and other which are resistant (Pap^r) to pediocin AcH. These results are presented here.

MATERIALS AND METHODS

Bacterial strains and pediocin production

Five pediocin AcH producing Ped. Acidilactici strain, consisting of four wild-type strains (H, E, F, M) and one transconjugant (LB42-923), were use from our stock

collection. The pediocin AcH production phenotype in these five strains is encoded 8.9 kb plasmid pSMB74 (Motlagh et al. 1992). Strains were grown in trypticase-glucose-yeast extract (TGE) broth at 37°C for h and heated at 95°C for 5 min. The heated culture broth were centrifuged and the supernatant liquids were adjusted to pH 5.0with sterile sodium hydroxide and use as sources of pediocin AcH. The preparation had about 18000 activity units (AU)/ml against the indicator bacterium , *Lactobacillus plantarum NCDO 955* (Biswas *et al.* 1991; Yang *et al*, 1992). In some studies a plasmidless, pediocin AcH sensitive wild strain, Ped. Acidilactici LB-42, was used (Ray *et al.* 1989b).

Isolation of pediocin nonproducer variants

Initially a single colony, forming a big zone of inhibition against a lawn of *lact. Plantarum* NCDO 955, was isolated for each pediocin AcH producing strain. Each strain was grown in TGE broth at 37°C for 16 h, and 20 <1 from each was inoculated in 5 ml of TGE broth containing 20 <g/ml acriflavine (Ray *et al.* 1989a; Kim *et al.* 1992). After 16 h at 37°C, 20 <g/ml acriflavine and the process was repeated three times. At the end of three transfer, the culture broths were serially diluted and aliquots from selected dilutions were pour plated with TGE agar (TGE broth + 1.5% agar). The plates were incubated for 16 h at 37°C and plates with 40 to 50 colonies were selected from isolation of pediocin nonproducer colonies. For this each plate was overlaid with 5 ml of melted and tempered TGE soft agar (TGE broth + 0.8 % agar) inoculated with 5 <1 (about 10^6 cells) of an overnight culture of *lact. Plantarum* NCDO 955 (Ray et al. 1989a; Kim et al. 1992). In another experiment cells from spontaneous nonproducing variants were similarly plated and examined for pediocine AcH producing cells.

Assay of pediocin sensitivity of nonoproducer variants

The cells from the nonproducer colonies were purified by streaking on a TGE-agar prepoured plate and a single colony ws culture in TGE broth. This culture was then used prepare a soft agar lawn as above and inoculated with 10<1 of pediocin AcH preparation. The plates were incubated at 37°C for 16 h and examined for the absence (resistant) or presence (sensitive) of zones of growth inhibition.

Plasmid DNA analysis

The pediocin AcH producing strains and their nonproducing variants were grown at 37°C in TGE broth containing 20mmol/l DL-threonine to an O.D. at 600nm of about 0,5. The cells were harvested and used for the isolation of plasmid by lysozyme-mutanolysine lysis procedure followed by purification by CsS1-density gardient centrifugation (Ray et al. 1989a, b). The purified preparation were run in agarose gels, stained with EtBr and photographed.

Conversion of pediocin sensitivity and resistance of cells

Five pediocin AcH sensitive variants, isolated from four pediocin producing strains and the strain LB-42, were grown in TGE broth containing 2000AU/ml of pediocin Ach at 37°C for 16 h for three successive transfers. Each time 10<1 of culture was transferred to 5 ml of fresh broth. Controls were grown in the absence of pediocin AcH. After the third transfer the cultures were streaked on TGE-agar plates and the plates were incubated at 37°C for 16 h. Twenty-five colonies from each plate were tested for resistance or sensitivity of pediocine AcH as indicated by the absence or presence of zone growth inhibition. Similarly, pediocin AcH nonoproducing but resistant variant cell from five strains (H, E, F, M, and LB-42) were grown successively through 10-transfers by transferring 10<1 to 5ml of TGEbroth and incubating at 37°C for 16 h. After each transfer a loopful of broth was streaked on TGE agar plates, the plates were incubated and 20 colonies from each were examined for their sensitivity or resistance to pediocin AcH.

RESULS AND DISCUSSION

All five pediocin AcH producing strains were immune to pediocin AcH (Pai$^+$) and the colonies inhibited the lawn of the indicator strain *Lact. Plantarum* NCDO 955 (Fig. 1). Previous study have report that the pediocin AcH production phenotype (Pap$^+$) is encoded in 8,9 kbplasmid pSMB74. When these strain were grown in the presence of acriflavine,

plated for isolation and examined against a lawn of Lact. Plantarum NCDO 955, some colonies were not surrounded by zone of growth inhibition and considered as pediocin AcH nonproducer.

FIG. 1 Pediocin AcH of Pediococcus acidilactici H colonies produced zone of growth of inhibition on a lawn of *Lactobacillus plantarum* NCDO 955 used as indicator. Similar obtain with strains E, F, M and LB42-923.

Among the five strains, strains F yielded higher percentage of nonproducer variants and strains M gave the least (Table 1). Also among the nonproducer variants most were resistant (Pap^r) to pediocin AcH, but a small percentage from each strain was sensitive (Pap^s) to it. In previous studies, isolation of pediocin AcH sensitive variants was reported only from strain H (Ray et al. 1989a; Kim at al. 1992). The inability to isolate sensitive variants from other strains could be due to examining only a few colonies of nonproducing variants. This could also account for the failure by others to isolated sensitive variants from the pediocin producing strains PAC 1.0 and SJ 1 (Gonzales and Kunka 1987; Schaved et al. 1992). A comparison of the palsmid profiles of the pediocin AcH producer strains and their nonproducer variants revealed that failure to produce pediocin AcH was due to loss of the 8-9 kb plasmid pSMB74 (fig. 2; Ray et al. 1989a, 1992; Kim et al. 1992; Motlagh et al. 1992).

Table 1. Frequency of pediocin-resistant and sensitive variants among nonproducers following curing treatment of pediocin AcH producing *Pediococcus acidilactici* strains*.

Producing strains	Number of nonproducin colonies out of total (%)	Number of sensitive out of nonprodu-cer (%)	% of sensiti-ve out of total
H	25/1436 (1.7)	4/25 (16.0)	0.3
E	22/1994 (1.1)	2/22 (9.1)	0.1
F	57/1128 (5.1)	1/30 (3.3)	0.1
M	12/2295 (0.5)	3/12 (25.0)	0.1
LB42-923	6/677 (0.9)	2/6 (33.3)	0.3

* Strains were grown at 37°C for 16 h TGE broth containing 20 < g/ml of acriflavine three successive transfer to isolated the nonproducing variants

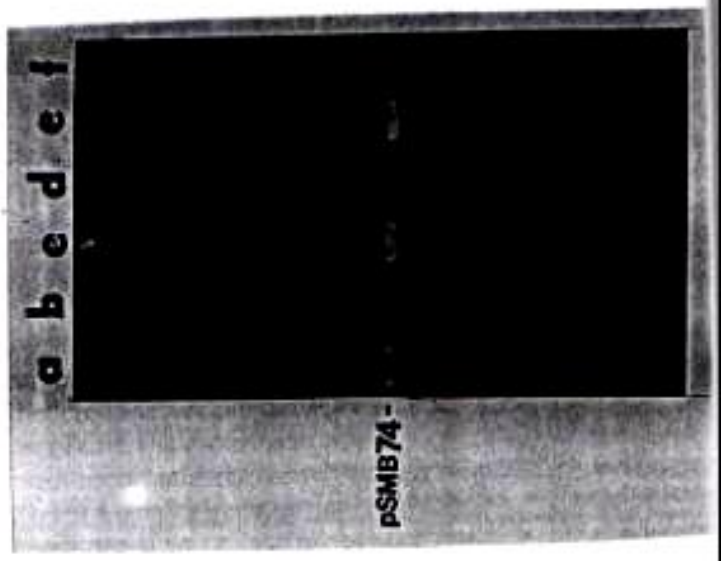

FIG. 2 plasmid profiles of pediocin AcH producing *Pediococcus acidilactici* strains and their plasmid cured nonproducer variants. Producer strains H, E, and (lane a, c, e, respectively) have the small plasmid, pSMB74, along with other large plasmid. In their nonproducer variants (lane b, d, f, respectively), pSMB74 is not present. Strain M and LB42-923 also had plasmid pSMB74 which was not present in their nonproducing variants (either resistant or sensitive to pediocin AcH). The numbers represent molecular weight of the two plasmid in kb. Ch, Chromosomal DNA.

It has been observed that a strain sensitive to pediocin AcH (Pap^-Pap^s) contains variants that are resistant (Pap^-Pap^r) to pediocin AcH (Hanlin et al. 1993). The sensitive cells were killed by pediocin AcH but the resistant cells survived. It is suspected that during plasmid curing study, loss of pSMB74 by some cells resulted in nonproducer variant (Pap^-) that were either resistant (Pap^r) or sensitive (Pap^s) to pediocin. However, the Pap^s cells were killed by pediocin AcH produced by the producer cells (pap^+) in the population, while the Pap^r cells survived, multiplied and were isolated (Table 1). The reason why the sensitive cells could be isolated, although at a low frequency, was probably that they were originally Pap^r cells but change to Pap^s during their purification and culturing in the absence of pediocin AcH prior to testing against pediocin.

This hypothesis was tested in two subsequent studies. In one study, sensitive variants isolated from five producer strains (H, E, M, LB42-923) and a naturally sensitive strain (LB-42), were grown in the presence or absence of pediocin AcH for three consecutive transfers. Atotal of 25 colonies for each variant were examined for sensitivity or resistance to pediocin AcH. All colonies growing in the absence of pediocin AcH were sensitive, but all became resistant when grown in the presence of pediocin AcH. (Table 2)). Thus, there were some naturally resistant variant cells in the population of each sensitive strain. The sensitive cells were killed by pediocin AcH but the resistant cells survived multiplied. This phenomenon also has been observed for sensitive strain growing in the presence of nisin (Hurst 1981; Hanlin et al. 1993).

In the second study, Pap^-Pap^r variants of strains H, E, F, M and LB-42 were grown for to consecutive transfer in the absence of pediocin AcH and after each transfer 20 colonies were examined for sensitivity to pediocin AcH. Result presented in Table 3 indicated that after the fifth transfer, sensitive cellwere isolated for all strain from among the 20 colonies examined. Their frequency, although it varied with strain, increased during subsequent transfers. Thus Pap^r to Pap^s. Transition resistant to sensitive phenotype has also reported for bacterial strains that were initially sensitivity to nisin but were resistant to it by growing in nisin (Hurst, 1981).

Table 2. Conversion of pediocin-sensitive *Pediococcus acidilactici* variants to pediocin resistance following growing in the presence of pediocin AcH.

Strains	Number resistance/number sensitive	
	Control	Pediocin AcH treated
H	0/25	25/25
E	0/25	25/25
F	0/25	25/25
M	0/25	25/25
LB42	0/25	25/25

* Strain were grown at 370°C for 16 h for three successive transfer in TGE broth without (control) or with 2000 Au/ml of pediocin AcH

During this study, we observe that some spontaneous Pap^-Pap^r variants isolated from the five Pap^+Pai^+ strains, retained the Pap^-Pap^r phenotype for long periods (beyond 10 consecutive transfer). We investigated the reason by plating the non producer variant and overlaying the colonies with a lawn of Lact. Plantarum NCDO 955. Surprisingly, we observed a few colonies in the population from such a variant that were Pap^+ (Fig. 3)

Table 3 Conversion of pediocine-resistant *Pediococcus acidilactici* to pediocin-sensitive variants during consecutive transfer and growth in the absence of pediocin AcH.

Strains	Number sensitive out of 20 colonies following transfer*									
	1	2	3	4	5	6	7	8	9	10
H	0	0	0	0	0	6	20	20	NT	NT
E	0	0	0	0	0	3	5	8	12	18
F	0	0	0	0	0	3	6	8	19	20
M	0	0	0	0	0	1	4	7	16	18
LB42	0	0	0	0	0	1	4	5	20	NT

*Strain were grown in TGE Broth at 370C for 16 h. After each transfer, a loopful of culture broth was streaked on the TGE agar plate, incubated and 20 colonies were tested for pediocin sensitivity.
NT, Not tested.

Examination of plasmid profiles also indicated a faint band of pSMB74 as compared to the thick band obtained from normal pediocin AcH producer (Fig. 4). For this study the same population of cells were used for pediocin AcH producer and nonproducer (but mixed) cultures. This canble verified by comparing the density of the large plasmid of

the producer strainsand the nonoproducer variant. This was because only a few harboured pSMB74 but all obtained the large plasmid

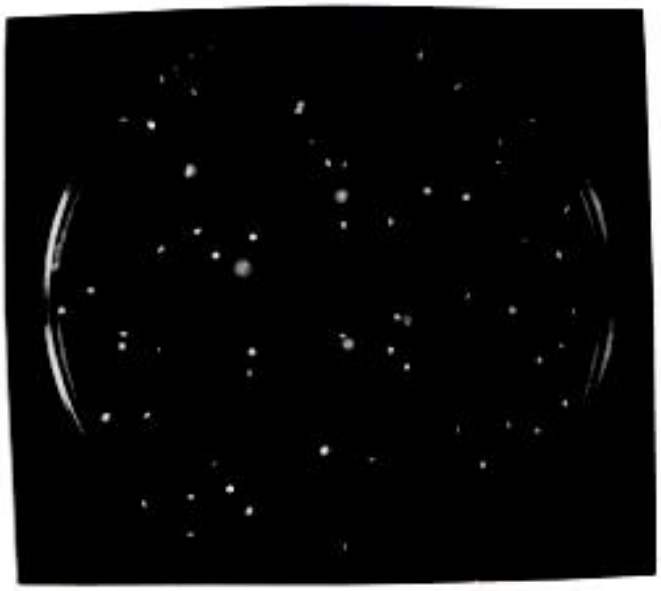

Fig. 3 Colonies for a spontaneous pediocin AcH-nonproducing variant from Pediococcus acidilactici H against a lawn of Lactobacillus plantarum NCDO 955.One colony (indicated by the arrow) had a clear zone of growth inhibition. The diffusion zone around other colonies is due to acid produced by Ped. Acidilactici H. Non producing variant from other strains also gave similar results.

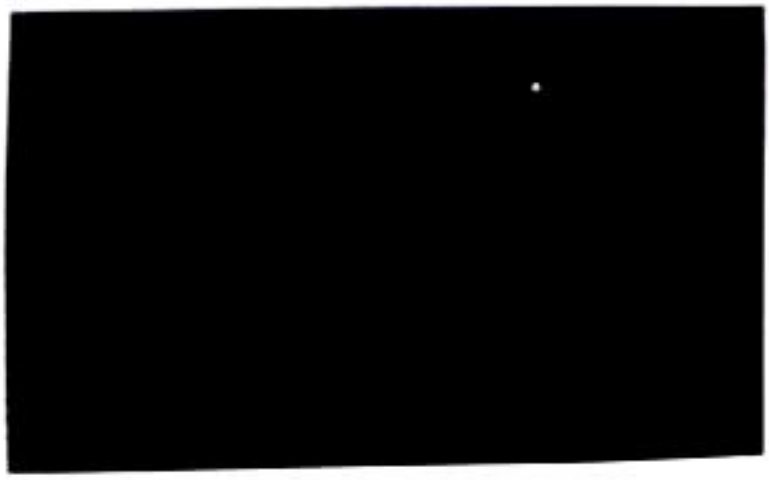

Fig. 4. Palsmid profile of pediocin AcH producing strain Ped. Acidilactici H (Lane a,c, d) and its spontaneous nonproducing variant (laneB). Palsmid pSMB74 was present in small amount in the nonproducer. For other explanation, see Fig. 2

We suspected that because only a few cells in a colony were producing pediocin AcH, it could not be detected by the normally used procedure of testing 5-20<1 of a broth culture against a lawn of indicator bacteria. However, cells in this population were constantly being exposed to a low dose of pediocin AcH, resulting in the survival only of the $Pap^{r}Pap^{s}$ cells.

Pediococcus acidilactici strains can be resistant to pediocin AcH due to several factors. A producer strain in immune to it (Pai^{r}) due to production of specific immunity protein produced by a gene encoded in pSMB74 (Johnson et al. 1992; Motlagh et al, 1992). The nonproducing variants can be resistant to it (Pap^{r}) due to non-specific reason. The variant cells bind pediocin AcH on the cell-wall surface like the producer cells. Their resistant could be due to the inability of pediocin AcH to pass through the wall, which is a prior requirement before pediocin AcH comes in contact with the cytoplasmic membrane to destabilize the structure in the sensitive cells (Ray 1992c, 1993). In addition *Ped. Acidilactici* cells have intracellular protease(s) that canhydrolyze and inactivate pediocin AcH (Johnson et al, 1992). However, the mechanism(s) by which many other Gram-positive bacterial strains are resistant to pediocin AcH is not known. Causes of resistance to nisin immunity protein; (b) non-specific immunity protein;(c) growing sensitive cells in the presence of nisin and (d) production of nisinase enzyme (Ray 1992b).

The result of this study have explained why a nonproducer variant, obtained following loss of specific genes, from a producer strain of *ped. acidilactici* became resistant to prediocin AcH while growing in the presence of pediocin AcH. The result also have shown how a resistant variant produced by growing a sensitive strain in the presence of pediocin AcH, reverted to sensitivity when grown in the absence of pediocin AcH. The last observation has important implication if bacteriocins are to be used as food biopreservatives. It suggests that the acquired resistance of bacterial cells to a bacteriocin that is produced by growing them in environment containing that bacteriocin, is a temporary trait. It is lost soon by growing them in an environment free of that bacteriocin.

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