COMPARATIVE ANALYSIS OF TWO METHODS FOR CURING *PED*$^+$ PLASMID PCP289 FROM *PEDIOCOCCUS ACIDILACTICI* MTCC 5101

Praveen Pal Balgir, Puja Bhatia, Pushpinder Kaur and Vipin Kumar*

Department of Biotechnology, Punjabi University, Patiala-147002, Punjab, India.
**Directorate of Research, SVPUA&T, Meerut, 250110 (UP), INDIA*
E-mail of corresponding author*: puja17bt@gmail.com*

Abstract: The DNA-intercalating agents, ethidium bromide and ascorbic acid (vitamin C), were used to eliminate plasmid DNA from *Pediococcus acidilactici* MTCC 5101, a lactic acid bacteria. The strain was grown in the presence of 0.03 mM ethidium bromide or 1 mM ascorbate for 18 hours at 42°C, which resulted in the loss of its ability to produce pediocin, a plasmid-specified trait. Agarose gel electrophoresis of plasmid DNA and spot-on-lawn assay showed concomitant loss of an 8.9 kb plasmid pCP289 coding for pediocin from these colonies. Since ascorbic acid is a readily available, shows less effect on cell viability, non-hazardous and more efficient compound in contrast to ethidium bromide, the possibility of its use in determining plasmid-encoded traits in food grade lactic acid bacteria is also proposed.

Key words: Curing, ethidium bromide, ascorbic acid, *Pediococcus acidilactici* MTCC 5101

INTRODUCTION

Pediococcus are of eminent importance because of their widespread use as starter cultures in food fermentation. Genus *pediococcus* belongs to the homo-fermentative gram-positive lactic acid bacteria (LAB). Additionally, they also possess an ability to produce antimicrobial peptides, commonly known as bacteriocins. Bacteriocins produced by *Pediococcus spp.* are designated as pediocins and are effective against many gram positive pathogenic and food spoilage bacteria, including *Listeria monocytogenes* (Daeschel & Klaenhammer 1985; Gonzalez & Kunka 1987; Bhunia *et al*., 1988; Pucci *et al.*, 1988). It is also reported that bacteriocin production is a plasmid linked character. Table 1 summarizes different *Pediococcus* strains and plasmids (pediocin+).

Plasmid encoded traits in LAB and in strains of pediococci, in particular, have been determined by conventional chemical curing agents. Antibiotics such as Novobiocin, Mitomycin C, Rifampin, Rifampicin and Flavophospholipol; Pefloxacin phenolic compounds and DNA intercalating dyes such as acridine orange, acriflavine, ethidium bromide and sodium dodecyl sulphate have been used as curing agents (Gonzalez & Kunka 1987; Ray *et al.*, 1989; Halami et al 1999; Jamal et al 2005). Alcoholic extract of *P. zeylanica*, an Indian medicinal plant, was used as curing agent (Beg & Ahmad 2000). Among other curing agents used by the authors, maximum plasmid curing was observed in case of pefloxacin (88%), followed by ethidium bromide (36%), acridine orange (14%) and alcoholic extract of *P. zeylanica* (14%).

However, the use of intercalating dye such as ethidium bromide is hazardous. It doesn't only cure plasmids, but in some cases generates plasmid deletions and loss of sporulation ability (Crameri *et al.*, 1986). It may also lead to rearrangement of the DNA molecule rather than actual loss of plasmid (Gupta & Batish 1992). Chromosome changes occur at high frequencies which result in phenotypic changes such as loss of enzyme activity, antibiotic resistance or ability to sporulate (Crameri *et al.*, 1986).

In addition to these intercalating dyes, ascorbic acid can also be used as a curing agent at elevated temperatures. Ascorbic acid (vitamin C) is a non-hazardous curing agent that is readily available and is safe to handle, resulting in approximately 35% curing rate in many strains of pediococci. (Ramesh *et al.*, 2000).

In the present study, demonstration of plasmid curing of Pediococcus acidilactici MTCC 5101 have been tried using two different methods, which were employed and compared for their efficiency of curing.

MATERIAL AND METHODS

P. acidilactici MTCC 5101 is a gram positive bacterium isolated from chilly pickle. This work has been carried out in Micobial Genetics Lab, Department of Biotechnology, Punjabi University, Patiala, India.

Curing experiment

A single colony of *P. acidilactici* MTCC 5101 showing a big zone of inhibition against *P.acidilactici* LB42 was inoculated into deMan, Rogosa and Sharpe (MRS) broth-M1164 (de Man *et al*., 1960) Hi media and incubated at 37°C for 18 hours. This culture was used as inoculum for the curing experiments.

Curing with ascorbic acid

The sodium salt of L-ascorbic acid (SRL, Mumbai) was used by preparing a 100 mM stock solution whose pH was adjusted to 7.2 by addition of $NaHCO_3$. MRS broth supplemented with ascorbic acid at 0.5 mM, 1.0 mM, 2.0 mM, 3.0 mM, 4.0 mM and 5.0 mM concentrations were inoculated with freshly grown 18 hour old culture of strain MTCC 5101 at 42°C**.**

Curing with ethidium bromide

0.25mm stock solution of ethidium bromide was prepared. MRS broth was supplemented with ethidium bromide at different concentrations (0.007 mM, 0.02 mM, 0.03 mM, 0.04 mM, and 0.05 mM). It was then inoculated with freshly grown 18 hour old culture of strain MTCC 5101 at 42°C.

Optical density of the culture broth was measured at 600 nm using a spectrophotometer (Thermospectronic, UV). Viability of cells was expressed as a percentage of control, where the cells were grown in MRS broth without the curing agents. Appropriate dilutions of the culture broth were spread plated in MRS agar and incubated for 24 hours at 37°C for colonies to appear. The colonies were then overlaid with *P.acidilactici* LB42 and checked for zones of inhibition around them. Colonies of the parental strain, which exhibited a zone of inhibition, and its cured derivative were purified individually, transferred into MRS broth and grown for 18 hours at 37°C.

Spot on lawn assay

Spot-on lawn assay was carried out by spotting 5 µl of CFS (Cell free supernatant) of parental and cured strain of *P. acidilactici* MTCC 5101 by both ethidium bromide and ascorbic acid on MRS (0.75% w/v) soft agar that was overlaid on MRS (1% w/v) bottom hard agar and seeded with approximately 10^6 cfu/ml of indicator strain *P. acidilactici* LB42. Assay plates were incubated at 37ºC for 24 hours (Coventry *et al*., 1995).

Isolation of plasmid DNA

Isolation of plasmid pCP289 was carried out using overnight grown broth cultures of parental and cured strain of *Pediococcus acidilactici* MTCC 5101. DL-threonine at a concentration of 10 µg/ml was added to culture, that induces protoplast formation, which became sensitive to lysozyme and SDS and induced cell lysis. The cells were harvested by centrifugation of 3 ml culture broths in eppendorfs for 5 min at 5000 rpm. For the isolation of plasmid DNA from *Pediococcus* isolate, standard protocol of Anderson & Mckay (1983) was followed with minor modifications. Plasmids were analyzed by agarose (1.0 %) gel electrophoresis, stained with ethidium bromide and photographed. Supercoiled Plasmid DNA Ladder (Bangalore Genei, India) was used as molecular size marker.

RESULT AND DISCUSSION

To determine whether plasmid curing by ethidium bromide or ascorbic acid are involved in sensitizing the resistant bacteria, the resistant organisms were treated with ethidium bromide or ascorbic acid, and analyzed for the disappearance of plasmid-DNA by spot-on- lawn test and isolation of plasmid.

Curing with ethidium bromide

The effect of ethidium bromide on the growth of *P. acidilactici* MTCC 5101 is shown in Figure-1. Curing was done at a concentration of 0.03 mM ethidium bromide. The viability of strain MTCC 5101 was 65% with 0.007 mM and 54% with 0.03 mM ethidium bromide after 18 hours of growth. However, it decreased drastically to 14% in the case of cells grown in the presence of highest concentration of ethidium bromide (0.05 mM), indicating that this concentration was close to the lethal dose. Therefore, toxic effect of ethidium bromide on the cells was clearly depicted.

Curing with ascorbic acid

The effect of ascorbic acid on the growth of *P.acidilactici* MTCC 5101 is shown in Figure 2. Curing with ascorbic acid was done at a concentration of 1 mM. The viability of strain MTCC 5101 was 95% with 0.5 mM and 88% with 1.0 mM ascorbic acid after 18 hours of growth. However, it decreased drastically to 16% in the case of cells grown in the presence of 5.0 mM ascorbic acid, indicating that this concentration was close to the lethal dose. Luxuriant growth of strain MTCC 5101 was, however, observed in MRS broth in the absence of ascorbic acid. The toxic effect of L-ascorbate on the cells was clearly dose-dependent.

Figure 3 shows the colonies of strain *P. acidilactici* MTCC 5101 grown in MRS broth and cured colonies. It can be seen that certain colonies (such as colony ‘r’) retained the ability to produce a zone of growth inhibition on a lawn of strain LB42, whereas some of the other colonies, like colony ‘c’ were unable to show any antagonism. Nearly 85-90% of the colonies lost the capacity to produce a zone of growth inhibition using either of the two methods.

Figure 4 shows zone of inhibition of *P. acidilactici* MTCC 5101 on lawn of LB42 while there is no zone that appeared on cured derivatives of ethidium bromide (E)

and ascorbic acid (A). Agarose gel electrophoresis revealed the elimination of a 8.9 kb plasmid pCP289 from the cured strain (Figure 5). This result substantiates the fact that ascorbic acid and ethidium bromide treated cells lost the plasmid pCP289 coding for pediocin production.

Ethidium bromide is a DNA intercalating agent which is believed to inhibit the synthesis of DNA (Bouanchaud, *et al.*, 1968). L-Ascorbic acid exhibits a wide array of biological functions, notably modifying the properties of DNA, generation of hydrogen peroxide and hydroxyl radicals by auto-oxidation and lipid peroxidation of membrane components (Shamberger, 1984). The reactive oxygen species such as OH^- and H_2O_2 are involved in DNA damage by a Fenton-type reaction which has been shown to occur in bacterial cells (Imlay & Linn., 1989). Strains of *P. acidilactici* do not produce catalase, which is otherwise known to be a protective enzyme preventing H_2O_2- and OH^--mediated DNA damage (Halliwell & Aruoma, 1991). This could probably account for the drastic loss of viability in ascorbate-treated cells of strain MTCC 5101.

Ascorbic acid-induced loss of plasmid-encoding antibiotic resistance has been demonstrated earlier in *Staphylococcus aureus* (Amabile-Ceuvas *et al*., 1991). Ascorbic acid causes conformational damage to unprotected circular covalently closed (CCC) plasmids (Morgan, 1976). This, in turn, results in an inefficient replication and may eventually lead to its loss during cell division.

Curing was done involving both methods, at concentrations of 0.03 mM ethidium bromide or 1 mM ascorbic acid (Figure 4), but a difference was seen on the viability of cells among the two treatments. Figure 6 shows that ascorbic acid leads to lesser effect on viability of cells as compared to ethidium bromide. At a 1 mM concentration of ascorbic acid, viability of cells was 88% in comparison to 0.03 mM concentration of ethidium bromide which showed 54% cell viability.

Besides this, the use of ascorbic acid as a curing agent circumvents the handling of chemical ethidium bromide, which is known to be mutagenic and carcinogenic. Additionally, ascorbic acid can also be used to eliminate the antibiotic resistant plasmids, an undesirable phenotype from LABs that are used as starter cultures in food and feed fermentation. Therefore, treatment with ascorbic acid is a more efficient method than treatment with ethidium bromide.

Table 1: *Pediococcus* strains and plasmids (pediocin+)

Producer Organism	Pediocin	Plasmid	Mol. Wt. of plasmid	Reference
P. pentosaceus ATCC 43200	Pediocin A	pFBB61,	13.6MD	(Piva & Headon, 1994)
P. acidilactici PAC 1.0, NRRL-5627	Pediocin PA-1	pRSQ11	9.3 kb	(Gonzalez & Kunka 1987; Marugg *et al.*, 1992)
P. acidilactici PC	Pediocin PC		8.47 kb	(Jager & Harlander, 1992)
P. acidilactici SJ-1	Pediocin SJ-1		4.6 MD	(Schved *et al*., 1993)
P. acidilactici PO_2	Pediocin PO_2		5.5 MD	(Coventry *et al*., 1995; Cho *et al*., 1996)
P. acidilactici H, E, F, M	Pediocin AcH	pSMB74	8.9 kb	(Bhunia *et al*., 1988; Bhunia *et al*., 1990; Motlagh *et al*., 1992; Motlagh *et al*., 1994)
Pediococcus sp. KCA1303-10	Pediocin K1		9.1 kb	(Kim *et al*., 2000)
Pediococcus pentosaceus pep1	pediocin P	pHD1.0	9.0 MD	(Osmanagaoglu *et al*., 2000)
P. parvulus AT077 *P. pentosaceus* S34 *Lb. plantarum* WHE92	pediocin AcH	pAT077 pS34 pWHE92		(Miller *et al*., 2005)
Pediococcus acidilactici MTCC 5101	pediocin CP2	pCP289	8.9 Kb	(Kaur & Balgir 2007)

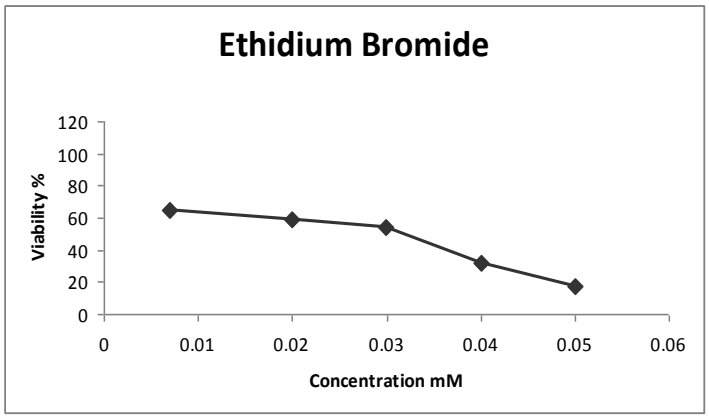

Figure 1: Effect of Ethidium Bromide on the viability of *P. Acidilactici* MTCC 5101.

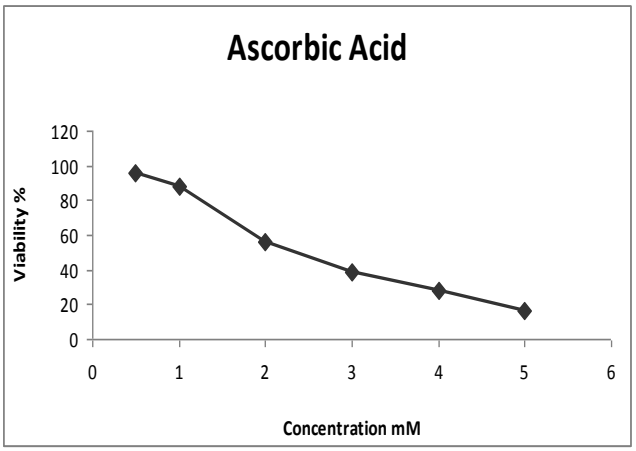

Figure 2: Effect of Ascorbic Acid on the viability of *P. acidilactici* MTCC 5101.

Figure 3: Colonies of the parental strain (colony r) and its cured derivative (colony c) obtained after treatment of *P. acidilactici* MTCC 5101 cells with curing agent.

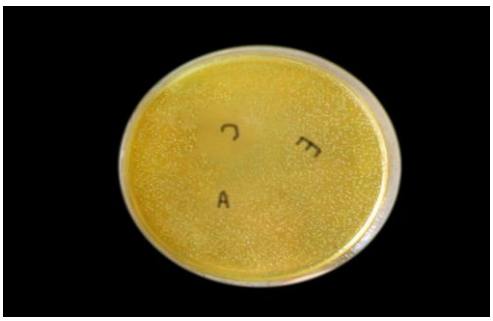

Figure 4: Spot-on-lawn assay performed on *P.acidilactici* MTCC 5101 showing zone of inhibition (C) on lawn of LB42. No zone appeared on cured derivatives of ethidium bromide (E) and ascorbic acid (A).

Figure 5: Plasmid analysis of strain *Pediococcus acidilactici* MTCC 5101 by 1.0% agarose gel electrophoresis. Lane a: Supercoiled Plasmid DNA Ladder (Bangalore Genei); Lane b: Plasmid profile of parental strain; Lane c: Plasmid profile of cured strain (EtBr) and Lane d: Plasmid profile of cured strain (AA). Arrows indicate loss of 8.9 kb plasmid from cured strain.

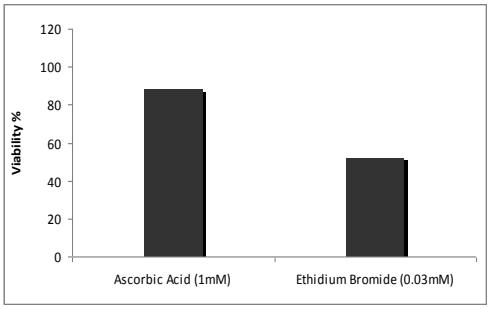

Figure 6: Viability comparison of Ascorbic Acid (AA) and Ethidium Bromide (EtBr) cured derivatives.

REFERENCES

AMABILE-CUEVAS, C.F.; PINA-ZENTELLA, R. and WAH-LABORDE, M.E. (1991). Decreased resistance to antibiotics and plasmid loss in plasmid-carrying strains of *Staphylococcus aureus* treated with ascorbic acid. Mutat Res, **264**: 119-25.

ANDERSON, D.G. and MCKAY, L.L. (1983). Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl Environ Microbiol **46**: 549-52.

BEG, A.Z. and AHMAD, I. (2000). Effect of Plumbago zeylanica extract and certain curing agents on multidrug resistant bacteria of clinical origin. World Journal of Microbiology and Biotechnology **16**: 841-844.

BHUNIA, A.K.; JOHNSON, M.C. and RAY, B. (1988). Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. *J Appl Bacteriol*, **65**: 261-8

BHUNIA, A.K.; JOHNSON, M.C.; RAY, B. and BELDEN, E.L. (1990). Antigenic property of pediocin AcH produced by *Pediococcus acidilactici* H. *J Appl Bacteriol*, **69**: 211-5.

BOUANCHAUD, D.H.; SCAVIZZI, M.R. And CHABBERT, Y.A. (1968). Elimination by ethidium bromide of antibiotic abbert. resistance in enterobacter and staphylococci. *J. Gen. Microbiol*, **54**: 417-425.

CHO, H.Y.; YOUSEF, A.E. and YANG, S.T. (1996) Continuous production of pediocin by immobilized *Pediococcus acidilactici* PO2 in a packed-bed bioreactor. *Applied Microbiology and Biotechnology*, **45**: 589-594.

COVENTRY, M.J.; MUIRHEAD, K. and HICKEY, M.W. (1995). Partial characterisation of pediocin PO2 and comparison with nisin for biopreservation of meat products. *Int J Food Microbiol*, **26**: 133-45.

CRAMERI, R., DAVIES, J. E., HUTTER, R. (1986) Plasmid curing and generation of mutations induced with ethidium bromide in streptomycetes. J Gen Microbiol 132: 819-24.

DAESCHEL, M.A. and KLAENHAMMER, T.R. (1985). Association of a 13.6-Megadalton Plasmid in *Pediococcus pentosaceus* with Bacteriocin Activity. *Appl. Environ. Microbiol*, **50**: 1538-1541

DE MAN, J.C.; ROGOSA, M. andSHARPE, M. E. (1960). A medium for the cultivation of Lactobacilli. *J. Appl. Bacl*., **23**: 130–135.

GONZALEZ, C.F. and KUNKA, B. S. (1987). Plasmid-Associated Bacteriocin Production and Sucrose Fermentation in *Pediococcus acidilactici*. *Appl. Environ. Microbiol*, **53**: 2534-2538.

GUPTA, R.K. and BATISH, V.K. (1992). Protoplast-induced curing of bacteriocin plasmid in *Lactococcus lactis subsp. lactis* 484. Journal of Applied Microbiology **73**: 337-341.

HALAMI, P.M.; CHANDRASHEKAR, A. and JOSEPH, R. (1999). Characterization of bacteriocinogenic strains of lactic acid bacteria in fowl and fish intestines and mushroom. Food Biotechnology **13**: 121-136.

HALLIWELL, B. and ARUOMA, O. I. (1991). DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. FEBS Lett **281**: 9-19.

IMLAY, J.A. and LINN, S. (1988). DNA damage and oxygen radical toxicity. Science 240: 1302-9 JAGER, K., HARLANDER, S. (1992) Characterization of a bacteriocin from *Pediococcus acidilactici* PC and comparison of bacteriocin-producing strains using molecular typing procedures. *Applied microbiology and biotechnology*, **37**: 631-637.

JAGER, K. and HARLANDER, S. (1992). Characterization of a bacteriocin from *Pediococcus acidilactici* PC and comparison of bacteriocin-producing strains using molecular typing procedures. *Applied microbiology and biotechnology*, **37**: 631-637.

JAMAL, H.; CHUA, K.H.; FREDERICK, D.; MAHMOOD AMEEN, A. and SALMAH, I. (2005). Plasmid DNA Analysis of Pasteurella Multocida Serotype B Isolated from Haemorrhagic Septicaemia Outbreaks in Malaysia. *Malaysian Journal of Microbiology*, **1**: 35-39.

KAUR, B. and BALGIR, P.P. (2007). Pediocin CP2 Gene Localisation to plasmid pCP289 of *Pediococcus acidilactici* MTCC 5101. *Internet J. Microbiol*. 3

KIM, C.H.; JI, G.E. and AHN, C. (2000). Purification and molecular characterization of a bacteriocin from *Pediococcus sp*. KCA 1303-10 isolated from fermented flat fish. *Food Sci. Biotechnol*, **9**: 270-276.

MARUGG, J.D.; GONZALEZ, C.F.; KUNKA, B.S.; LEDEBOER, A.M.; PUCCI, M.J.; TOONEN, M.Y.; WALKER, S.A.; ZOETMULDER, L.C. and VANDENBERGH, P.A.(1992). Cloning, expression, and nucleotide sequence of genes involved in production of pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0. *Appl. Environ. Microbiol,* **58**: 2360-7.

MILLER, K. W., RAY, P., STEINMETZ, T., HANEKAMP, T., RAY, B. (2005). Gene organization and sequences of pediocin AcH/PA-1 production operons in *Pediococcus* and *Lactobacillus* plasmids. *Lett. Appl. Microbiol*, **40**: 56-62.

MORGAN, A.R.; CONE, R.L. and ELGERT, T.M. (1976). The mechanism of DNA strand breakage by vitamin C and superoxide and the protective roles of catalase and superoxide dismutase. *Nucleic Acids Res.*, **3**: 1139-49.

MOTLAGH, A.; BUKHTIYAROVA, M. and RAY, B. (1994). Complete nucleotide sequence of pSMB 74, a plasmid encoding the production of pediocin AcH in *Pediococcus acidilactici*. Letters in applied microbiology, **18**: 305-312.

MOTLAGH, A.M.; BHUNIA, A.K.; SZOSTEK, F.; HANSEN, T.R.; JOHNSON, M.C. and RAY, B. (1992). Nucleotide and amino acid sequence of pap-gene (pediocin AcH production) in *Pediococcus acidilactici* H. *Lett Appl Microbiol*, **15**: 45-8.

OSMANAGAOGLU, O.; BEYATLI, Y.; GUNDUZ, U. and SACILIK, S.C. (2000). Analysis of the genetic determinant for production of the pediocin P of *Pediococcus pentosaceus* Pep1. *J. Basic Microbiol*, **40**: 233-41.

PIVA, A. and HEADON, D.R. (1994). Pediocin A, a bacteriocin produced by *Pediococcus pentosaceus* FBB61. Microbiology **140** (Pt 4): 697-702.

PUCCI, M.J.; VEDAMUTHU, E.R.; KUNKA, B.S. and VANDENBERGH, P.A. (1988). Inhibition of *Listeria monocytogenes* by using bacteriocin PA-1 produced by *Pediococcus acidilactici* PAC 1.0. *Appl. Environ. Microbiol*, **54**: 2349-53.

RAMESH, A.; HALAMI, P.M. and CHANDRASHEKAR, A. (2000). Ascorbic acid-induced loss of a pediocin-encoding plasmid in *Pediococcus acidilactici* CFR K7. *World Journal of Microbiology and Biotechnology*, **16**: 695-697.

RAY, S.K.; JOHNSON, M.C. and RAY, B. (1989). Bacteriocin plasmids of *Pediococcus acidilactici. Journal of industrial microbiology*, **4**: 163-171.

SCHVED, F.; LALAZAR, A.; HENIS, Y. and JUVEN, B.J. (1993). Purification, partial characterization and plasmid-linkage of pediocin SJ-1, a bacteriocin produced by *Pediococcus acidilactici*. *J. Appl. Bacteriol*, **74**: 67-77.

SHAMBERGER, R.J. (1984). Genetic toxicology of ascorbic acid. Mutat Res, **133**: 135-59.