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# Heterogeneity of macrolide-lincosamide-streptogramin phenotype & conjugal transfer of *erm*(B) in *Pediococcus pentosaceus*

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***Background & objectives*: *Pediococcus pentosaceus* has been reported to cause clinical infections while it is being promoted as probiotic in food formulations. Antibiotic resistance (AR) genes in this species are a matter of concern for treating clinical infections. The present study was aimed at understanding the phenotypic resistance of *P. pentosaceus* to macrolide-lincosamide-streptogramin B ($MLS_B$) antibiotics and the transfer of AR to pathogens.**

***Methods*: *P. pentosacues* isolates (n=15) recovered from fermented foods were screened for phenotypic resistance to $MLS_B$ antibiotics using disc diffusion and microbroth dilution methods. Localization and transferability of the identified resistance genes, *erm*(B) and *msr*(C) were evaluated through Southern hybridization and *in vitro* conjugation methods.**

***Results*: Four different phenotypes; sensitive (S) (n=5), macrolide (M) (n=7), lincosamide (L) (n=2) and constitutive ($cMLS_B$) (n=1) were observed among the 15 *P. pentosaceus* isolates. High-level resistance (>256 µg/ml) to $MLS_B$ was observed with one $cMLS_B$ phenotypic isolate IB6-2A. Intermediate resistance (8-16 µg/ml) to macrolides and lincosamides was observed among M and L phenotype isolates, respectively. Cultures with S phenotype were susceptible to all other antibiotics but showed unusual minimum inhibitory concentration (MIC) values of 8-16 µg/ml for azithromycin. Southern hybridization studies revealed that resistance genes localized on the plasmids could be conjugally transferred to *Enterococcus faecalis* JH2-2.**

***Interpretation & conclusions*: The study provides insights into the emerging novel resistance patterns in *P. pentosaceus* and their ability to disseminate AR. Monitoring their resistance phenotypes before use of MLS antibiotics can help in successful treatment of Pediococcal infections in humans.**

**Key words** Antibiotic resistance - conjugation - *Pediococcus pentosaceus*

*Pediococcus pentosaceus* is a facultative anerobe, homofermentative, Gram-positive cocci belonging to the lactic acid bacteria (LAB) and widely used in food fermentations[1-3]. The role of *P. pentosaceus* in causing infections is a matter of debate as it is often isolated with other organisms or causes mild infections[4]. It has been identified as an opportunistic pathogen in immunocompromised patients[5] and this pathogenic

trait of *P. pentosaceus* can create challenges in treating human infections as these are intrinsically resistant to glycopeptide group of antibiotics[4]. With the emerging reports on the detection antibiotic resistance (AR) genes in food-borne bacteria, the safety of commercially available LAB cultures has become a prerequisite with AR[6]. The anticipated problem is that foodborne bacteria might contain naturally occurring AR genes that could be ultimately transferred to human pathogens in the gut when ingested[3]. European Food Safety Authority also recommends that only probiotic strains which do not show resistance to antibiotics are considered safe for human and animal purpose[7].

Although new antibiotics have been evolved, of macrolide-lincosamide-streptogramin B ($MLS_B$) superfamily antibiotics are still in use for human infections and as animal growth promoters[8,9]. Hence, the development of resistance to these antibiotics is a cause of concern[8,10]. In addition, various novel resistance phenotypes being observed in pathogenic[11] and food-borne[12] bacterial species have complicated the treatment of human infections.

Acquired resistance to erythromycin (ERY) among LAB species such as *Lactobacillus* and *Enterococcus* along with human pathogens[8] has been described. Further, the treatment of $MLS_B$-resistant human pathogens are difficult to treat due to the emergence of novel mechanisms evident through unusual phenotypes[11,12]. Although ERY resistance ($ER^r$) is commonly observed among food LAB[8], but *Pediococcus* species has shown no resistance to ERY among *P. pentosaceus* isolates[1,13-15]. In our previous study conducted on ERY and tetracycline (TET) resistance genes evaluated by PCR in LAB from various fermented foods, we have reported the presence of *erm*(B), *msr*(C) and several *tet* genes in *P. pentosaceus* cultures[16]. This study was undertaken to characterize the phenotypic $MLS_B$ heterogenicity, determine the localization and transferability of the identified resistance genes [*erm*(B), *msr*(C)] in *P. pentosaceus* isolates.

## Material & Methods

*Bacterial isolates & growth conditions*: Fifteen *P. pentosaceus* isolates obtained from naturally fermented food (*idli* and *dosa* batter), dairy product-curd (*dahi*) and fermented dry sausage[16] identified by biochemical and molecular characterization were included in this study. The *E. faecalis* strain JH2-2 was donated by Dr Charles MAP Franz, Federal Research Centre for Nutrition and Food, Institute of Hygiene and Toxicology, Karlsruhe, Germany. All the isolates were grown at 37°C in deMan, Rogosa and Sharpe (MRS) and/or brain heart infusion (BHI) broth or agar (HiMedia, Mumbai, India). Bacterial pure cultures were maintained in 40 per cent glycerol at −80°C. The isolates were sub-cultured twice before use. These experiments were conducted at Microbiology & Fermentation Technology department, CSIR-Central Food Technological Research Institute, Mysuru.

*Antibiotic susceptibility*: The minimum inhibitory concentrations (MICs) for clindamycin (CLD), azithromycin (AZI), lincomycin (LIN), spiramycin (SPI), clarithromycin (CLA) and pristinamycin (PRI) were determined by microbroth dilution method in MRS medium[17]. The plates were incubated at 37°C in ambient air. MICs of CLD were also defined in the presence of sub-inhibitory concentrations of ERY[12]. A double disc diffusion test with CLD (2 µg) and ERY (15 µg) discs was performed on MRS agar as described earlier[12]. The CLD resistant ($CLD^R$) isolates were subjected to modified Hodge test for detection of lincosamide nucleotidyltransferase (*Lnu*) activity[12]. All the antibiotics either in powder form or discs were procured from HiMedia.

*Isolation of DNA & detection of clindamycin resistance gene by PCR*: The genomic DNA from test cultures was isolated using phenol-chloroform method as described earlier[18]. The isolated DNA was used as a template in PCR amplification reaction for *lnu*(A) and *lnu*(B) gene using the primers *lnu*(A)-F-5'- GGTGGCTGGGGGGTA GATGTATTAACTGG-3' and *lnu*A -R-5'-GCTTCTTTTGAAATACATGGTATTTT TC GATC-3' or *lnu*B-F-5′-CCTACCTATTGTTTGAA-3′ and *lnu*B-R-5′-ATAAC GTTAC TCTCCTATTC-3′[19]. The reaction mixture was composed of 5 pmol of each forward and reverse primers, 0.2 mM dNTPs mix (Bengaluru GeNei), 1× PCR buffer (Sigma-Aldrich, USA), 2.5 mM $MgCl_2$ (Merck, Bengaluru), 4 µl containing 10 ng of genomic DNA as template and 1 µl containing 3U of *Taq* DNA polymerase (Sigma). PCR analysis was carried out as follows: 35 cycles of amplification including 30 sec of denaturation at 94°C, 40 sec of annealing at 44°C (*lnu*A) and 55 °C *lnu*(B) and 40 sec of elongation at 72°C.

*Plasmid isolation & Southern hybridization*: Isolation and purification of plasmids were performed[12] and

plasmids were analyzed on 0.7 per cent agarose gel. The separated plasmids were transferred to a Hybond-N + Nylon membrane (Sigma) using the downward capillary transfer technique[12]. The DNA bound to the membrane was hybridized with purified PCR products of *erm*(B) and *msr*(C), labelled with digoxygenin. Hybridization and detection were performed according to the manufacturer's recommendations (Roche Chemicals, Germany).

*Conjugation experiment & evaluation of transconjugants*: The transferability of $ER^r$ genes was determined in *P. pentosaceus* isolates, by *in vitro* conjugation using filter mating as described earlier[20]. Two isolates, IB4-2A and IB6-2A with M and $cMLS_B$ phenotypes respectively, were selected as donor isolates and the *E. faecalis* JH2-2 [resistant to rifampicin (RIF)] as the recipient. Conjugation experiments were performed with the donor/recipient ratios of 1:1, 10:1 and 50:1. At the end of the mating period, cells from the membrane filters (plate mating) were scraped off and suspended in 1 ml of the peptone physiological saline solution. Serial ten-fold dilutions of the above suspensions were prepared, and appropriate dilutions were spread onto BHI agar plates containing ERY (10 µg/ml) and RIF (50 µg/ml) as selective agents. The plates were incubated at 37°C and examined for colonies (transconjugants) after 24, 48 and 72 h. The donor and the recipient cell counts were enumerated by plating the dilutions on appropriate selective medium for donor (10 µg/ml of ERY) and recipient (50 µg/ml of RIF). The frequency of the resistance transfer was evaluated by the number of transconjugants/donor cells. The mating experiments were conducted three times in duplicates, and an average of the frequency values was reported. Transconjugants obtained were verified by comparing their fingerprints with that of donor and recipient isolates obtained by random amplified polymorphic DNA (RAPD)-PCR using M-13 primers employing a colony PCR approach[12].

## Results

The *P. pentosaceus* isolates were subjected to CLD (2 µg) and ERY (15 µg) double disc test to determine their resistance phenotype. Among the 15 isolates tested, four distinct phenotypes were detected (Table). Five isolates were assigned to sensitive (S) phenotype which exhibited larger zones around ERY and CLD disks (Fig. 1). In contrast, one isolate showed no zone of inhibition for both the antibiotics and was designated $cMLS_B$ phenotype. Other two discrete non-inducible phenotypes observed were M and L phenotype (Fig. 1). Seven isolates with M phenotype showed intermediate sensitivity to CLD and resistance to ERY. Two isolates designated L phenotype displayed resistance to CLD and intermediate sensitivity to ERY.

The distribution of MIC values for the tested antibiotics is summarized in the Table. High-level resistance to all the antibiotics was observed for the isolate IB6-2A, the sole $cMLS_B$ phenotypic strain. Isolates with S phenotype showed susceptibility to all the antibiotics but displayed MIC values of 8-16 µg/ml toward the synthetic 15-membered ring macrolide, AZI. Isolates with M phenotype demonstrated resistance to all macrolides while remained susceptible to lincosamides. In comparison, isolates with L phenotype showed resistance to lincosamides as well

**Table.** Macrolides, lincosamides and streptogramin B phenotypes and minimum inhibitory concentrations (µg/ml) among *Pediococcus pentosaceus*

| Phenotype | *n* | AZI | CLA | CLI | LIN | SPI | PRI |
|---|---|---|---|---|---|---|---|
| $E^s$ $C^s$ | 5 | 8-16 | <1 | <1 | <1 | 2 | <1 |
| M | 7 | 8 | 2-4 | <1 | <1 | 8-16 | <1 |
| L | 2 | 8 | 2-4 | 8-16 | 16 | 8 | <1 |
| cMLSB | 1 | >256 | >256 | >256 | >256 | >256 | >256 |

$E^S$ $C^S$, erythromycin and clindamycin sensitive; M, macrolide; L, lincosamide; cMLSB, constitutive-macrolide, lincosamide and streptogramin B phenotype; AZI, azithromycin; CLA, clarithromycin; CLI, clindamycin; LIN, lincomycin; SPI, spiramycin; PRI, pristinamycin

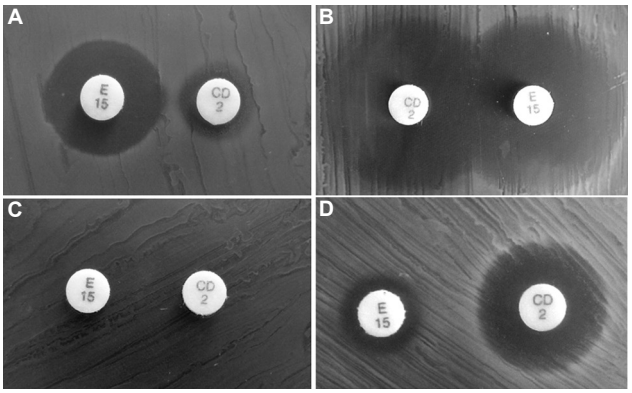


**Fig. 1.** Evaluation of phenotypic $MLS_B$ heterogenicity in *Pediococcus pentosaceus* isolates. The double disc diffusion was performed using erythromycin disc ER15 (15 µg) and clindamycin disc CD 2 (2 µg) (**A**) Lincosamide (L) phenotype [Erythromycin sensitive ($ERY^S$) and cindamycin intermediate resistant ($CLD^{IR}$)]; (**B**) Sensitive (S) phenotype ($ERY^S$ and $CLD^S$); (**C**) $cMLS_B$ phenotype ($ERY^R$ and $CLD^R$); (**D**) M phenotype: ($ERY^{IR}$) and ($CLD^S$); $MLS_B$, macrolide-lincosamide-streptogramin B; $cMLS_B$, constitutive-macrolide, lincosamide and streptogramin B phenotype.

as low-level resistance to macrolides and spiramycin. Pristinamycin was found to be active against all isolates except, IB6-2A. In addition to the MIC test performed, the resistance profile of CLD was evaluated for the M phenotype isolates (n=7) after treating them with sub-inhibitory concentrations (induction) of ERY. However, no changes in the MIC values were observed upon induction.

*Pediococcus pentosaceus lack lnu(A) or lnu(B) gene*: To evaluate the genetic background of CLD resistance observed in isolates with L (n=2) and $cMLS_B$ (n=1) phenotypes, the antibiotic inactivation test was performed using CLD-sensitive *Micrococcus luteus* ATCC 9341. None of the cultures showed characteristic cloverleaf inhibition zone. In addition, the test isolates did not show any positive PCR amplification for either *lnu*(A) or *lnu*(B) genes.

*Localization of erm(B) & msr(C) genes*: From *P. pentosaceus* cultures, the plasmids demonstrated their presence with different sizes ranging from 1.3 to >21 kb (Fig. 2A). Southern hybridization was performed with *erm*(B) and *msr*(C). Although the PCR results were positive for *erm*(B) and *msr*(C) in 12 and eight isolates respectively[16], the Southern hybridization studies resulted positive for *erm*(B) and *msr*(C) only in two isolates (IB4-2A and IB6-2A) (Fig. 2B) with the isolated plasmids.

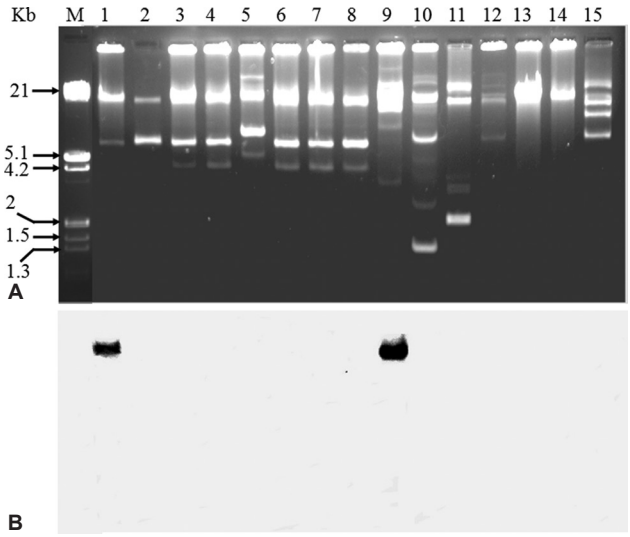


**Fig. 2.** Southern hybridization with undigested plasmids of *Pediococcus pentosaceus* isolates against *erm*(B) gene probes. (**A**) Plasmids; (**B**) Southern blot analysis of plasmids hybridized with internal segments of *erm*(B) gene obtained by PCR and labelled with digoxigenin. Lane M: HindIII digested λ-DNA marker; lanes 1 to 15: *P. pentosaceus* isolates IB4-2A, IB4-2B, IB3-3, 8, B-1, IB3-1A, IB3-1B, IB3-2, IB6-2A, DB3-E4, DB3-STR3B, CHS-3E, 3, 4, IB2-1, respectively. The isolates IB4-2A and IB6-2A in lanes 1 and 8 respectively were positive for *erm*(B). The same isolates were also found positive for *msr*(C) with similar localization (data not shown). The localization of these genes was found on high molecular weight plasmids.

*Transfer of erm(B) to Enterococcus faecalis JH2-2*: Among all the *P. pentosaceus* isolates, two cultures (IB4-2A and IB6-2A) confirmed for *erm*(B) and *msr*(C) were investigated for their ability to transfer the genes to *E. faecalis* JH2-2 by *in vitro* conjugation. Among the three-different donor/recipient ratios, conjugation was observed with 50:1 combination and was successful with the sole $cMLS_B$ isolate, IB6-2A with a transfer frequency of $1 \times 10^{-6}$/ donor cells. Confirmation of the transconjugant's identity was carried out by RAPD-PCR analysis revealing a banding pattern similar to that of the recipient and completely different from the donor isolate (data not shown). Genotypic characterization of the transferred resistance markers in transconjugants by PCR showed positive results only for *erm*(B). Plasmid profiling of the recipient (*E. faecalis* JH2-2) showed no transfer of plasmid from the donor isolate. The PCR results of *erm*(B) gene transfer was substantiated with Southern hybridization results when conducted with the digested chromosomal DNA of the recipient (Fig. 3). These results indicated the possible association of *erm*(B) gene with transposons. The $ER^r$ transfer was

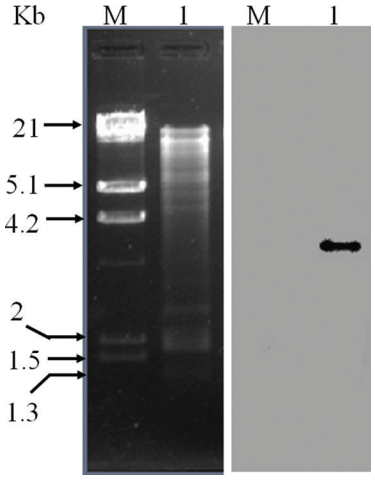


**Fig. 3.** Localization of *erm*(B) gene in transconjugant *Enterococcus faecalis* JH2-2 and antibiotic transferability of *P. pentosaceus*. Southern blot analysis of the total genomic DNA from *E. faecalis* JH2-2 digested with *Bam*HI and hybridized with internal fragment of *erm*(B) gene obtained by PCR and labelled with digoxigenin. Lane M: HindIII digested λ-DNA marker; lane 1: *Bam*HI digested total genomic DNA of transconjugant *E. faecalis* JH2-2 after conjugation (left). Positive Southern hybridization of *erm*(B) probe with the digested DNA fragment (right) indicating the transfer of *erm*(B) gene from *P. pentosaceus* IB6-2B isolate to *E. faecalis* JH2-2 during conjugation.

also validated with the performance of 'D' zone test where, all the selected transconjugants showed $cMLS_B$ phenotype and presented higher MIC values (128 µg/ml) for ERY.

## Discussion

Acquisition of $ER^r$ methylase genes (*erm* gene) confers resistance to $MLS_B$ antibiotics[21]. Expression of $MLS_B$ resistance can be constitutive ($cMLS_B$) or inducible ($iMLS_B$). In the later, the mRNA becomes active in presence of lower concentrations of macrolides and induces resistance to CLD. By contrast, in constitutive expression, active methylated mRNA is produced even in the absence of an inducer[10]. The $cMLS_B$ resistant phenotype was evident with one isolate that showed no zones of inhibition around the two antibiotic discs used. This characteristic phenotype was substantiated with high-level resistance to all the MLS antibiotics used.

*P. pentosaceus* has been shown to be susceptible to MLS antibiotics[14]. In our study, the five *P. pentosaceus* isolates with S phenotype were expected to be sensitive to all $MLS_B$ antibiotics considered. However, discrepancies were observed between this sensitive phenotype and MIC values for AZI (8-16 µg/ml). Further evaluation of genes that confer resistance specific to macrolides need to be investigated.

Besides the target modification, active efflux (due to drug pumps) which reduces intracellular antibiotic concentration is characterized with 14- and 15- membered macrolide resistance[20]. This was found to be one of the major resistances to macrolide antibiotics and associated with M phenotype[22,23]. To verify that M phenotype observed was not due to altered $iMLS_B$ phenotype, the CLD MIC was determined in the presence of sub-inhibitory concentrations of ERY. The results indicated unaltered CLD MICs which confirmed M phenotype in *P. pentosaceus* isolates. In contrast to M phenotype, two isolates displayed L phenotype that was comparable to that shown in *Enterococcus* species in our previous study[12]. However, the CLD inactivation test specific for LIN resistance genes was negative. Such results may be due to unidentified resistance genes prevailing in bacteria or due to changes in the existing *erm* genes[11].

The double disc diffusion test (D zone test) being a useful guide to interpret the presence of MLS resistance genotypes[24] is restricted to only pathogens and not for investigations in either LAB or probiotics. Previous studies conducted on MLS resistance in pathogenic bacteria reported that the disc diffusion test could be used to predict the genotype[25]. This was evident in our earlier study where one isolate with $cMLS_B$ was found to harbour *erm*(B)[16]. However, CLD not being an inducer or a substrate for efflux pump[10], the two L phenotype isolates did not show any CLD inactivation phenotype or *lnu*(A) or *lnu*(B) genes. Such complexity of CLD resistance and ERY sensitive phenotype was observed previously in enterococci[12] and in *Staphylococcus agalactiae*[11]. *Pediococcus* isolates frequently exhibit one or more ATP-binding cassette-type multidrug resistance[26]. Thus, it could be hypothesized that the observed phenotype with unexplained resistance patterns could be due to other efflux pump mechanisms or undefined novel mechanisms.

In LAB, including *P. pentosaceus*, plasmid associated resistance genes possibly spread to other more harmful bacteria[5]. This was evident with the transfer of *erm*(B) gene from IB6-2B to *E. faecalis* JH2-2 indicating the considerable potentiality of *P. pentosaceus* isolates in transferring AR genes to human pathogens. Although plasmids could be identified in these test isolates, we could detect the localization of resistance genes on plasmids only in two isolates. These results showed that majority of the isolates carried the *erm*(B) and *msr*(C) genes on the chromosomal DNA and suggested their association with transposons. More insights on this aspect could be gained by considering a large number of isolates from various sources. The same isolates although were found positive for TET resistance genes in our preliminary study[16], we could not determine the localization of the *tet* genes in their genome. These findings provide emerging complex condition in characterization of AR and thus depend on basic phenotypic tests.

In conclusion, the results of our study supported the presence of diverse MLS resistance mechanisms justified by rapid variations in resistance phenotypes. The disc diffusion along with susceptibility tests may facilitate in identifying novel MLS resistance mechanisms. Monitoring of resistance phenotypes before the use of MLS antibiotics can help in effective and successful treatment of pediococcal infections.

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***Conflicts of Interest*:** None.

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