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Conjugal transfer of *aac*(6′)Ie-*aph*(2″)Ia gene from native species and mechanism of regulation and cross resistance in *Enterococcus faecalis* MCC3063 by real time-PCR

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ABSTRACT

High level aminoglycoside resistance (HLAR) in the lactic acid bacteria (LAB) derived from food animals is detrimental. The aim of this study was to investigate the localization and conjugal transfer of aminoglycoside resistance genes, *aac*(6′)Ie-*aph*(2″)Ia and *aph*(3′)IIIa in different *Enterococcus* species. The cross resistance patterns in *Enterococcus faecalis* MCC3063 to clinically important aminoglycosides by real time PCR were also studied. Southern hybridization experiments revealed the presence of *aac*(6′)Ie-*aph*(2″)Ia and *aph*(3′)IIIa genes conferring HLAR in high molecular weight plasmids except in *Lactobacillus plantarum*. The plasmid encoded bifunctional *aac*(6′)Ie-*aph*(2″)Ia gene was transferable from *Enterococcus avium* (n = 2), *E. cecorum* (n = 1), *E. faecalis* (n = 1) and *Pediococcus lolii* (n = 1) species into the recipient strain; *E. faecalis* JH2-2 by filter mating experiments thus indicating the possible risks of gene transfer into pathogenic strains. Molecular analysis of cross resistance patterns in native isolate of *E. faecalis* MCC3063 carrying *aac*(6′)Ie-*aph*(2″)Ia and *aph*(3′)IIIa gene was displayed by quantification of the mRNA levels in this study. For this, the culture was induced with increasing concentrations of gentamicin, kanamycin and streptomycin (2048, 4096, 8192, 16384 μg/mL) individually. The increasing concentrations of gentamicin and kanamycin induced the expression of the *aac*(6′)Ie-*aph*(2″)Ia and *aph*(3′)IIIa resistance genes, respectively. Interestingly, it was observed that induction with streptomycin triggered a significant fold increase in the expression of the *aph*(3′)IIIa gene which otherwise was not known to modify the aminoglycoside. This is noteworthy as streptomycin was found to confer cross resistance to structurally unrelated kanamycin. Also, expression of the *aph*(3′)IIIa gene when induced with streptomycin, revealed that bacteria harbouring this gene will be able to overcome streptomycin bactericidal action at specific concentrations. HLAR in *E. faecalis* MCC3063 may be due to the combined expression of both the *aac*(6′)Ie-*aph*(2″)Ia and *aph*(3′)IIIa genes which could be therapeutically challenging. A combined expression of both the genes in *E. faecalis* MCC3063 may yield HLAR which could be therapeutically challenging. The study highlights the significant alterations in the mRNA expression levels of *aac*(6′)Ie-*aph*(2″)Ia and *aph*(3′)IIIa in resistant pathogens, upon exposure to clinically vital aminoglycosides.

1. Introduction

Bacteria evolve and adapt to the antibiotic rich environment by several mechanisms [1]. Under constant antibiotic threat, transmission of acquired resistance to sensitive bacteria increases [2]. Antibiotic resistance genes are frequently spread all around in the environment [3] even where antibiotic usage has been minimal such as wildlife [4]. Aminoglycoside resistance in commensal bacteria isolated from food source is a growing concern as aminoglycosides are used in animal husbandry. The widespread aminoglycoside resistance gene, *aac*(6′)Ie-*aph*(2″)Ia was reported in lesser known species like *Enterococcus cecorum* an emerging pathogen [5] and *Pediococcus lolii* derived from farm animals [6]. Among the commensal bacteria, lactic acid bacteria (LAB) are regarded to be potential source for the spread of resistance genes [7,8]. Since, *Enterococcus* spp. are known to acquire as well as transfer antibiotic resistance genes easily [9] there is a high risk on its introduction in the environment and its association with mammals [10].

Antibiotic resistance is mostly associated with conjugative plasmids and transposons that propagate between commensal

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bacteria and pathogens [11]. The transfer of antibiotic resistance genes among different genus was observed *in vitro* and *in vivo* demonstrations [12–14]. Aminoglycoside resistance genes have been previously found on plasmids, transposons and insertion elements [13]. The presence of these genes on the plasmids and mobile genetic elements facilitates its rapid spread upon selective pressure of antibiotics in the farm environment [15–17]. In the case of aminoglycoside resistance in lactic acid bacteria, experiments on horizontal gene transfer of gentamicin, streptomycin and kanamycin resistance have been carried out *in vitro* [18–20]. *In vitro* and *in vivo* experiments by Lester et al. [21], demonstrated co-transfer of erythromycin and gentamicin bifunctional gene into a sensitive *E. faecium*. *In vivo* demonstration of high level aminoglycoside resistance gene, *aac*(6′)Ie-*aph*(2″)Ia transfer into *Escherichia coli* were observed in mice [9].

The transfer of aminoglycoside resistance genes in LAB is overwhelming owing to the therapeutic importance of aminoglycoside drugs in human medicine and is scarcely documented. In case of the human gut, the risks are higher when the individual is on antibiotic treatment [22]. Aminoglycoside class of drugs is structurally similar and confers resistance by inactivation of the drug by aminoglycoside modifying enzymes. Selection with one aminoglycoside can stimulate adaptive resistance to other aminoglycosides [18]. Similarly, challenging the enterococci isolates with gentamicin and kanamycin may initiate horizontal gene transfer.

The phenomenon of inducible resistance in other class of antibiotics such as macrolides and glycopeptides are well characterized [23] however such mechanisms in aminoglycosides are yet to be understood completely. Upon aminoglycoside treatment with doses lower than minimum inhibitory concentration (MIC) values, there appears to be higher induction in transcription rates of the modifying genes as one gene has the ability to modify a set of antibiotics in this class [24]. Also, it has been predicted that challenging with one aminoglycoside can make bacteria resistant to other aminoglycosides of similar structure [25]. For instance, constant assault with kanamycin resulted in cross resistance to tobramycin which have a similar structure [25]. Thus it was concluded that cross resistance in aminoglycosides are observed within the same class among structurally related antibiotics. In contrast, cross resistance between apramycin and gentamicin which are structurally unrelated were also observed in normal flora isolated from farm animals which were under apramycin administration [26]. The regulation and the mechanism of cross resistance in aminoglycosides have not been explicitly investigated however, recent studies throw some light on the riboswitch mechanism of induction in aminoglycoside modifying genes. Jia et al. [24], describes the transcription initiation or induction of the aminoglycoside modifying *aac/aad* genes found in Gram-negative organisms due to structural changes in 5′ leader mRNA on aminoglycoside binding. The leader mRNA of both *aac/aad* gene have conserved sequences [24] and were experimentally shown to change the secondary structure upon interaction with specific 4,6 deoxystreptamine group of aminoglycosides. Exact mechanism of regulation for the propagation and expression of resistance genes are unavailable for the 4, 5 deoxystreptamine and APH enzymes which phosphorylates 4,6 deoxystreptamines [27].

In the present study, localization of the aminoglycoside resistant genes such as *aac*(6′)Ie-*aph*(2″)Ia and *aph*(3′)IIIa in different *Enterococcus* spp. derived from food animals have been carried out by Southern hybridization. Further, *in vitro* filter mating experiments were conducted to assess horizontal gene transfer of the *aac*(6′)Ie-*aph*(2″)Ia gene via conjugation by lesser known species such as *E. cecorum, E. avium* and *P. lolii* which is the first report. In addition, mRNA expression levels and cross resistance patterns of the *aac*(6′)Ie-*aph*(2″)Ia and *aph*(3′)IIIa gene detected in *E. faecalis* MCC3063 were investigated upon induction with high concentrations of gentamicin, kanamycin and streptomycin individually.

2. Materials and methods

2.1. Bacterial isolates and culture conditions

LAB cultures, *E. faecalis* MCC3063 and *E. faecalis* RE-25 were used as a positive control for aminoglycoside resistance genes [*aac*(6′)Ie-*aph*(2″)Ia] and [*aph*(3′)IIIa] in Southern hybridization experiments, respectively. *E. faecalis* JH2-2 were obtained from Prof. Franz, C.M.A.P, Federal Research Centre for Nutrition and Food, Institute of Hygiene and Toxicology, Karlsruhe, Germany. *E. faecalis* JH2-2 was used as a recipient for conjugation experiments in this study. The cultures *E. cecorum* I40a, *E. faecalis* MCC3063, *E. avium* MCC2772, *E. avium* CS32+ and *P. lolii* MCC2972 were stored in glycerol stocks at −20 °C and grown in Lactic acid susceptibility medium (LSM) composed of 90%Isosensitest broth (Oxoid, Hamshire, England) +10% de Mann, Rogosa and Sharpe media (Himedia, Mumbai, India). These LAB isolates were used to detect *in vitro* transfer of the *aac*(6′)Ie-*aph*(2″)Ia and *aph*(3′)IIIa genes and have been previously characterized for the presence of these genes [6].

2.2. Plasmid isolation and southern hybridization

Aminoglycoside resistant LAB isolates (n = 22) were subjected to plasmid isolation [28]. Plasmid profiling of selected LAB cultures for the presence of high and low molecular weight plasmids was analyzed on a 0.7% agarose gel in 1X TAE buffer at a voltage of 60 V for 4–5 h. The plasmids were captured on a Hybond-N + Nylon membrane (Sigma-Aldrich) by downward capillary blotting as described earlier [29]. The amplified PCR product of the bifunctional aminoglycoside resistance genes *aac*(6′)Ie-*aph*(2″)Ia in *E. avium* MCC2772 and *aph*(3′)IIIa in *E. faecalis* CS11+ were purified by the gel elution kit (Sigma Aldrich, Bangalore) and labelled with DIG high prime (Roche, Biochemicals, Mannheim, Germany), dUTP antigen (4 μl) by overnight incubation at 37 °C. These probes were stored at −20 °C for further use. Southern hybridization, stringency washes and chromogenic detection was performed [30] as per manufacturer's instructions. The standard strain, *E. faecalis* RE-25 was used as a positive control for kanamycin resistant gene.

2.3. In vitro conjugation studies

Filter mating experiments were carried out to evaluate the transferability of gentamicin [*aac*(6′)Ie-*aph*(2″)Ia] and kanamycin [*aph*(3′)IIIa] resistance genes from *E. cecorum* I40a, *E. faecalis* MCC3063, *E. avium* MCC2772, *E. avium* CS32+ and *P. lolii* MCC2972 into the recipient strain *E. faecalis* JH2-2. The donor cultures were subjected to gentamicin and kanamycin induction (32 μg/mL) individually prior to filter mating. Conjugation experiments were carried out according to Sasaki et al., [31]. The donor: recipient ratios for each isolate are shown in Table 2. The transconjugants were selected on lactic acid bacteria susceptibility medium containing gentamicin (50 μg/mL) and rifampicin (50 μg/mL) or kanamycin (1024 μg/mL) and rifampicin (50 μg/mL). The donor and the recipient cells were grown on a selective medium supplemented with gentamicin (512 μg/mL) and rifampicin (50 μg/mL), respectively. The transfer frequency with respect to donor and recipients were calculated by the number of transconjugants per number of donors and recipient cells, respectively. The filter mating experiments were carried out three times in duplicates and averages of the frequency of transfer are listed in Table 2.