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Antibiotic resistance in lactococci and enterococci: phenotypic and molecular-genetic aspects

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Abstract

Extensive use of antibiotics in medicine, veterinary practice and animal husbandry has promoted the development and dissemination of bacterial drug resistance. The number of resistant pathogens causing common infectious diseases increases rapidly and creates worldwide public health problem. Commensal bacteria, including lactic acid bacteria of genera *Enterococcus* and *Lactococcus* colonizing gastrointestinal and urogenital tracts of humans and animals may act as vehicles of antibiotic resistance genes similar to those found in pathogens. Lactococci and enterococci are widely used in manufacturing of fermented products and as probiotics, therefore monitoring and control of transmissible antibiotic resistance determinants in industrial strains of these microorganisms is necessary to approve their Qualified Presumption of Safety status. Understanding the nature and molecular mechanisms of antibiotic resistance in enterococci and lactococci is essential, as intrinsic resistant bacteria pose no threat to environment and human health in contrast to bacteria with resistance acquired through horizontal transfer of resistance genes. The review summarizes current knowledge concerning intrinsic and acquired antibiotic resistance in *Lactococcus* and *Enterococcus* genera, and discusses role of enterococci and lactococci in distribution of this feature.

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Introduction

Antibiotic resistance in bacteria as a global medical problem

Antibiotic resistance has received increasing attention of scientists and society as an impediment to effective therapy of several diseases (1). After introduction of antibiotics in the 1940s, drug resistant bacteria were found soon (2). When new classes of antibiotics were discovered, the new types of resistance emerged in microorganisms, instigating fears that control of the curbed diseases might be lost. The reason for the rapid expansion of drug resistant bacteria in recent years is widespread use of antibiotics in human and veterinary medicine, stock breeding, agriculture. The dissemination of drug resistance is also determined by long application record of antibiotics as farm stock growth promoters (3-7).

Bacteria acquire antibiotic resistance by several ways: by horizontal transfer of plasmids carrying antibiotic resistance determinants, by recombination of foreign DNA into the chromosome, or by mutations in different chromosomal loci (8-10). Mutation rates in bacteria are quite low (~0.003 per genome per cell division), hence foundation for fast spread of antibiotic resistance among microorganisms is horizontal gene transfer (HGT) (11-13).

For several decades studies on the dissemination of antibiotic resistance have been focused on pathogenic bacteria, but recent investigations aroused speculations that commensal bacteria may act as reservoirs of antibiotic resistance genes and can transfer resistance determinants to pathogenic bacteria. From this point of view, the food chain is the main vehicle of antibiotic resistant bacteria with a direct link between animal gut microbiota and the human digestive tract (14-18). Lactic acid bacteria are common members of the human and animal intestinal tract widely used as starter cultures in food industry

and as probiotics. This heterogeneous microbial group can be regarded as potential host of antibiotic resistance genes with the risk of their transfer to commensal and pathogenic bacteria (8, 19-21).

This review summarizes the current knowledge on origin, distribution and mechanisms of antibiotic resistance in lactic acid bacteria of *Lactococcus* and *Enterococcus* genera with special emphasis on their role in the dissemination of antibiotic resistance in humans and animals.

Genera *Lactococcus* and *Enterococcus* and their applications

The genera *Lactococcus* and *Enterococcus* consist of Gram-positive cocci with low G+C content (34-42 mol% for enterococci, 38-40 mol% for lactococci) (22). They are non-spore-forming, facultatively anaerobic, catalase and oxidase negative, homofermentative bacteria showing complex nutritional requirements. For many years lactococci and enterococci were affiliated to the same genus *Streptococcus* as serogroups D (enterococci) and N1 (lactococci) (23). Only in 1984 Schleifer and Kilpper-Balz using DNA-DNA and DNA-rRNA hybridization technique provided strong evidence that *Streptococcus faecalis* and *Streptococcus faecium* should be classified as separate genus *Enterococcus* (24). Lactic streptococci were also transferred into a new genus *Lactococcus* (25). These genera can be easily distinguished due to ability of enterococci to grow at 45°C and in presence of 6.5% NaCl (26).

Bacteria of the genera *Lactococcus* and *Enterococcus* colonize gastrointestinal and urogenital tracts of humans and animals, inhabit the surface of plant leaves, fruits and vegetables and have high industrial importance (27-30). Lactococci and enterococci are widely used as starter cultures in manufacturing of fermented meat, dairy and vegetable products and as silage inoculant in fodder production (31-32). These microorganisms contribute a lot to the taste and flavor of fermented food, physical and chemical properties of raw milk and meat (33). Many strains of *Lactococcus lactis* produce bacteriocin nisin used in food and pharmaceutical industries for its ability to prevent growth of a broad spectrum of pathogenic and spoilage bacteria (34). Lactococci and enterococci are vital constituents of probiotic products due to high survival rate during gastrointestinal transit and proved beneficial health effect on the consumers (35-36).

Antibiotic resistance in lactococci and enterococci

General aspects

Industrial and probiotic strains of *L. lactis* and *Enterococcus faecium* interact with commensal microbiota and probably pathogens during their transit through human gastrointestinal tract and consequently they may be involved in dissemination of antibiotic resistance genes among them (37-39). In general, the mechanisms used by lactic acid bacteria for neutralization of devastating effects of antibiotic treatment are enzymatic inactivation or degradation of antibiotics, mutational alteration of antibiotic target, restricted import of antibiotics, activation of drug efflux systems that export antibiotics from bacterial cell (40-42). Intrinsic and acquired antibiotic resistance can be the result of combined work of all four mechanisms (43). All multidrug resistance transporters are divided into 2 superfamilies: utilizing energy of ATP hydrolysis (ATP binding cassette (ABC) superfamily) or utilizing the proton motive force (PMF) for drug expulsion and functioning as drug/H^+ or drug/Na^+ antiporters (44-48). To date 4 classes of multidrug efflux systems have been described for bacteria: (i) the major facilitator superfamily (MFS), (ii) the small multidrug resistance family (SMR), (iii) the resistance-nodulation-cell-division family (RND), (iv) the multidrug and toxic compound extrusion family (MATE) (49). Transporters of the RND and MATE family are distributed in gram-negative bacteria (5, 50).

Enterococci possess a broad spectrum of antibiotic resistance due to intrinsic and acquired mechanisms. Natural for these bacteria resistance to aminoglycosides arose from a low cellular permeability for this group of antibiotics. The insusceptibility to glycopeptides discovered in enterococci in 1980s is the main reason for application of avoparcin as feed additive for animals (31, 51-52). Most strains of *Enterococcus* exhibit resistance to erythromycin, tetracycline and chloramphenicol owing to presence of drug resistance genetic determinants (53-55). Quinolone-insusceptable enterococci were also detected. Resistance to quinolones could be related to a mutation in subunit of DNA gyrase, acquired property for synthesizing proteins of gyrase protection and using multidrug resistance efflux pumps EmeA and EfrAB (56). Much attention of scientists has been attracted to the problem of wide distribution of vancomycin resistance in enterococci. By 2007 more than 90% of *E. faecium* isolates in North America and 71% in Europe were resistant to vancomycin, and only 6% of *E. faecalis* isolates in North America and 25% in Europe were vancomycin-resistant (57-59). Noteworthy that for a long time these microorganisms were believed to be harmful to humans, as pathogenic strains of *E. faecalis* and *E. faecium* cause nosocomial infections such as urinary tract infections, hepatobiliary sepsis, wound infections, bacteremia, neonatal sepsis and endocarditis in individuals with depressed immune status (60-61). Grave menaces may arise from nosocomial vancomycin-resistant enterococci acting as intermediaries for mobile resistance genes of more deleterious pathogens, including *Staphylococcus aureus* (29, 62). Enterococci have an adjustment to capture and disseminate antibiotic resistance genes. One of the reasons for this feature is lack of CRISPR (clustered regularly interspaced short palindromic repeats) elements, preventing the acquisition of foreign DNA fragments (63).

In contrast to enterococci, lactococci are regarded as susceptible to the majority of antibiotics. Bacteria from *Lactococcus* genus are considered to have only low level intrinsic resistance to some antibiotics, such as colistin, fosfomycin, pipemidic acid and rifamycin, due to presence of multidrug efflux pumps (64-68).

Intrinsic antibiotic resistance in genera *Lactococcus* and *Enterococcus*

Intrinsic resistance to specific antibiotic is an inherent characteristic of bacterial species or genus, not subject to horizontal transfer and posing no risk in commensal bacteria. The defense mechanisms of intrinsic antibiotic resistance are generally related to presence of low affinity target, the absence of target, decreased uptake or efflux of drug. Actually, probiotic strains of lactococci and enterococci with intrinsic resistance to commonly used antibiotics could be even helpful in restoring gut microbiota after drug therapy.

According to the data of genome analysis, *L. lactis* IL1403 contains 40 ATP-dependent drug transporters, but only few of them have been characterized functionally (69-71). The best studied are LmrA, LmrP and LmrCD, encoded by chromosomal genes *lmrA*, *lmrP*, *lmrC* and *lmrD,* respectively. LmrA (**l**actococcal **m**ultidrug **r**esistant protein **A**TP) is a bacterial homolog of human multidrug resistance P-glycoprotein belonging to ABC superfamily (5, 70, 72). This multidrug transporter functions as a homodimer and realizes resistance to aminoglycosides, cephalosporins, macrolides, penicillins, quinolones, streptogramins and tetracyclines (73). Genome sequencing revealed homologs of LmrA in different genera of bacteria: *Oenococcus oeni*, *Escherichia coli*, *Bacillus subtilis*, *Helicobacter pylori*, *Mycobacterium genitalium*, *Haemophilus influenza*, *S. aureus* (74-77). LmrP belongs to the MFS family and confers resistance to a broad range of clinically important antibiotics: lincosamides, tetracyclines, streptogramins, 14- and 15-membered macrolides. This integral protein with 12 membrane-spanning segments functions as a drug/H^+ antiporter extruding drugs in exchange for protons (78-79). It pumps out cationic dyes, daunomycin, tetracyclines, and macrolides (50, 80). Homologs of LmrP were found in the genome sequences of *B. subtilis* (Bmr, Blt), *S. aureus* (Smr) and *Streptococcus pneumonia* (PmrA) (81-82). LmrC and LmrD are two half-transporters forming a heterodimeric ABC transporter. The experimental data suggested that expression levels of *lmrCD* genes were upregulated while antibiotics were added, in contrast to *lmrP*, *lmrA* and other genes of multidrug resistance transporters (69).

Enterococci possess multidrug transporters providing resistance to the variety of antibiotics. Transporter EfrAB in *E. faecalis* structurally and functionally related to LmrCD of *L. lactis* determines resistance to acriflavine, norfloxacin, ciprofloxacin, doxycycline, 4′,6′-diamidino-2-phenylindole (DAPI), and TPP chloride (75, 83). Another enterococcal drug efflux pump EmeA is a homolog of lactococcal LmrP transporter (84-85).

Clinical and food isolates of *E. faecalis* and *E. faecium* have significantly different resistance profiles. Isolates of *E. faecium* from pre-antibiotic era were susceptible to erythromycin, framycetin, streptomycin, penicillin, gentamicin, tetracycline and chloramphenicol (86). Enterococci are naturally resistant to cephalosporins because of penicillin-binding protein encoded by gene *pbp5* located on chromosome (87-88). Some species of *Enterococcus* genus, such as *E. gallinarum*, *E. casseliflavus E. flavescens,* possess intrinsic low level resistance to vancomycin. They carry gene located exclusively on chromosome that determines VanC-type of resistance. Another five types encoded by *vanA*, *vanB*, *vanD*, *vanE* and *vanG* genes correspond to acquired resistance. It should be noted that enterococci with VanC-type of resistance are susceptible to teicoplanin, unlike those with acquired transferable VanA-type of resistance (83, 89-90). Not only genes of vancomycin resistance may have plasmid or chromosomal localization. Genes coding for several enzymes with phospho- and acetyl-transferase activity involved in aminoglycosides resistance are located on plasmids and chromosome (91).

As opposed to enterococci, *L. lactis* strains exhibit sensitivity to most clinically important antibiotics: amikacin, ampicillin, first generation of cephalosporins, chloramphenicol, erythromycin, gentamicin, imipenem, oxacillin, penicillin, piperacillin, sulfonamide, tetracycline, trimethoprim/sulfomethoxazol, vancomycin. However, it should be noted that literature reports of antibiotic resistant phenotype in *L. lactis* strains appear with increasing frequency (10, 92-93).

Acquired antibiotic resistance in genera *Lactococcus* and *Enterococcus*

Enterococci and lactococci are able to acquire antibiotic resistance through mutations or HGT. Chromosomal mutations can result in increased resistance to antibiotics in different ways. The most frequent are mutations in genes coding for the drug target molecules, altering the antibiotic-binding site. Mutations in regulators or regulatory regions can contribute to antimicrobial resistance by leading to the overproduction of either intrinsic resistance determinants, such as efflux pumps or the target itself, which may overcome total inhibition by the drug. The HGT phenomenon occurs due to transformation, transduction and conjugation with acquisition of drug resistance determinant as a component of mobile genetic elements (94–96). Conjugation is a major mechanism of HGT in Gram-positive cocci, therefore rapid spread of antibiotic resistance in lactococci and enterococci is generally contributed by the conjugative plasmids and transposons (97–100). These extrachromosomal elements have a broad host spectrum and may be transferred to pathogenic bacteria of *Streptococcus*, *Staphylococcus* and other genera (101). Insertion sequences (ISs) can also have impact on the level of antibiotic resistance in bacteria by effecting the expression or transcription of certain genes, including silent genes. IS elements may be localized on the chromosome or/and plasmids and many of them carry complete promoters for antibiotic resistance genes. For example, IS elements of *E. faecium* influence glycopeptide resistance (102). Integrons or transposons are also involved in spread of antibiotic resistance among bacteria (103).

Acquired antibiotic resistance is widely distributed in bacterial species inhabiting human and animal bodies, where they contact with antibiotics and selection of resistant strains occurs naturally. In this case, lactococci and enterococci may act as reservoirs and vehicles for antibiotic resistance genes involved

in their dissemination to potential and obligate pathogens (3, 104-105). The transfer of resistance determinants takes place owing to broad host range plasmids of Gram-positive bacteria belonging to the *Streptomyces, Leuconostoc*, *Listeria, Lactococcus* genera (106-107). This group of plasmids includes pAMβ1 (encoding MLS – **m**acrolides, **l**incosamides, **s**treptogramin B resistance), pAM830 (MLS, vancomycin resistance), pRE25 (chloramphenicol, MLS resistance) from *E. faecalis* and pIP816 (vancomycin resistance), pRUM (chloramphenicol, streptothricin, streptomycin, MLS) from *E. faecium* (108). Acquired antibiotic resistance in enterococci is mainly connected with plasmid-located genes. It was shown that pAMβ1 could be transferred from *E. faecalis* into the plasmid-free strain of *L. lactis* and from *L. lactis* to mice intestinal bacteria during filter mating (109). Furthermore, nonconjugative plasmid pAMαl bearing *tet*-genes is co-resident and can be transferred with four conjugative plasmids pAMβ1, pAMδI, pAMδ2 and pAMδ3 (110). Sequence analysis of plasmid pRE25 from *E. faecalis* demonstrates the presence of antibiotic resistance genes closely related to those of *Streptococcus pyogenes*, *Staphylococcus agalactiae*, *S. aureus*, *B. subtilis*, *Campylobacter coli*, *Clostridium perfringens*, *Clostridium difficile* and even to the fish pathogenic lactic acid bacterium *Lactococcus garvieae* (111-112). The enterococcal *ermAMR* and *ermB*-like genes coding for erythromycin resistance are located on plasmid. The product of these genes assigns methylation of adenine at position 2058 of the 50S rRNA and determines MLS resistance. The resistance genes from *erm* family are disseminated among the members of *Enterococcus* genus (113). Tetracycline resistance in enterococci is connected to presence of different *tet*-genes: *tetM*, *tetS*, *tetO*, *tetK*, *tetL*. Products of the latter two genes constitute efflux proteins. Enzymes encoded by genes *tetM, tetS*, *tetO* change the ribosomal conformation and prevent the association of tetracyclines on ribosomes (114-116). It should be noted that drug-specific efflux pumps such as tetK and tetL are transmissible, as their genetic determinants are located on plasmid, while MDR efflux systems are usually encoded by chromosomal genes (8). Numerous similar genetic elements responsible for tetracycline resistance were found in *E. faecalis* (Tn*916*, Tn*918*, Tn*920*, Tn*925*, Tn*2702*), *E. faecium* (Tn*5031*, Tn*5032*, Tn*5033*, Tn*5233*) and *L. lactis* (Tn*5276*, Tn*5301*) (39, 110). Broad host range transposons of Tn*1545* family are common in enterococci and determine their resistance to tetracycline, erythromycin and kanamycin (115).

Another group of extrachromosomal elements is represented by pheromone-responding conjugative plasmids that also play a role in acquisition of antibiotic resistant phenotype by lactic acid bacteria. For example, 65-kb plasmid pCF10 of *E. faecalis* provides tetracycline resistance, pAM368 and pMG2200 carry vancomycin resistance determinants (108).

Transposons Tn*1545,* carrying *erm*, *tet*, and *aph-3'* genes, Tn*1546* carrying *vanA* gene cluster, Tn*916* and Tn*916*-type containing *tetM* gene, are frequently found among members of *Enterococcus* genus (3, 101). Transposon Tn*917* involved in dissemination of MLS resistance is localized on conjugative plasmid pAD1 of *E. faecalis* and could insert into the chromosome of recipient cells (60, 117). Genes *vanH, vanA, vanX* determining resistance to vancomycin and teicoplanin are located on the transposon Tn*1546* carried by plasmid pIP816, which replication region is identical to that of pAMβ1 (83, 108).

IS elements also play important role in dissemination of antibiotic resistance. Genes encoding β-lactamases are associated with IS*CR1* and have been detected on plasmids and integrons. One of these genes is $bla_{\text{CTX-M2}}$ found in isolates of *E. faecium,* although this gene is typical for gram-negative bacteria (102). Chloramphenicol and erythromycin resistance in *E. faecalis* is controlled by pRE25 conjugative plasmid (91).

It was shown that genes of antibiotic resistance such as *tetM* and *ermAM* may be transferred from *E. faecalis* to *L. lactis* and other bacteria, including pathogens *S. aureus* and *Listeria innocua* by conjugation in filter mating experiments (118). In 1997 Perreten et al. (86) isolated streptomycin-, tetracycline- and chloramphenicol-resistant strain *L. lactis* subsp. *lactis* K214 from soft cheese. Genes *str*, *cat* and *tet* encoding antibiotic resistance of the strain were found on plasmid pK214. Three proteins, products of these genes, are almost identical to the streptomycin-inactivating adenylase, chloramphenicol acetyltrasferase from plasmids of *S. aureus* and tetracycline resistance protein from *Listeria monocytogenes*. The gene *mef214* encoding drug efflux pump and probably derived from *E. coli* was also detected on plasmid pK214 of *L. lactis* subsp. *lactis* K214 (104). The low GC content of plasmid pK214 means that this genetic element could be transferred from other organisms with a lower GC% than lactococci. According to the analyses of sequence data, the plasmid is composed of distinct DNA segments found in *E. faecalis* and *E. faecium*. Experiments *in vitro* demonstrated that plasmid pK214 could be successfully transformed into *Streptococcus mutans* (105). *In vivo* transfer of *vanA* genes from *E. faecium* isolated from chicken to intestinal enterococci in human volunteers was demonstrated. In 2002 vancomycin-resistant *S. aureus* was isolated from the patients infected with vancomycin-resistant enterococci in the United States. According to the data of sequence analysis, the resistance was encoded by gene *vanA* from enterococci and acquired through the transposon Tn*1546* (119). In this case, normal microbiota, starter cultures in fermented food and probiotics play a role in the dissemination of antibiotic resistance genes (49).

Antibiotic resistance and safety of probiotic strains of lactococci and enterococci

One of the most important criteria for selection of bacterial strains for use in probiotics and food industry is the safety concern. In Europe, according to the Qualified Presumption of Safety (QPS) approach, the nature of any antibiotic resistance determinant present in a candidate microorganism should be identified.

L. lactis has a GRAS-status (generally recognized as safe) deserved by its safety, widespread application and extensive use in

food industry (120). Until recently, lactococci have been known to be sensitive to the majority of antibiotics. It appears now that these microorganisms are able to host broad range plasmids, transposons and facilitate prevalence of antibiotic resistance, which in turn provokes certain risks. A serious problem now is emergence and dissemination of antibiotic-resistant enterococci, causing nosocomial infections, endocarditis and infections of urinary tract (121-122). Probability of transmission of antibiotic resistance genes from gram-positive enterococci to gram-negative bacteria is very low *in vitro*, but transfer of plasmid pIP501 from *E. faecalis* to *E. coli* in native environment was recorded (105). Thus animals and humans serve as reservoirs where selection of antibiotic-resistant strains and transfer of drug resistance genes occur. Raw meat and milk products act as vehicles for the transmission of bacteria with antibiotic resistance determinants (123–125). To stop the spread of antibiotic resistance we should not neglect precautions about prudent use of antibiotics and cook with utmost care raw products that may be contaminated with enterococci.

Conclusion

As a result of widespread application of antibiotics in both human and animal treatment drug resistance rapidly disseminates among bacteria due to acquisition and spread of resistant genes. Since lactic acid bacteria are present in the gastrointestinal tract in large amounts and are widely used as probiotics and starter cultures in food industry, concerns have been raised about antibiotic resistance in these beneficial bacterial species. Lactococci and enterococci may serve as reservoirs of antibiotic resistance genes and transfer these genetic determinants to pathogenic and opportunistic bacteria in food products and gastrointestinal tract. In order to eliminate or minimize this possibility, antibiotic resistance of each industrial strain from *Enterococcus* and *Lactococcus* genera should be scrupulously examined on phenotypic and genotypic levels. Evaluation of molecular mechanisms underlying the horizontal transfer of antibiotic resistance genes in *Lactococcus* and *Enterococcus* species is essential to control their spread in the environment via the food chain.

Conflict of interest statement

The authors declare no conflict of interest.

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