Whole genome analysis as a tool for the safety assessment of antibiotic resistance in food-processing bacteria

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Introduction

Antimicrobial agents are one of the main therapeutic tools to protect humans and their domesticated animals from a variety of infections. However, the overuse of antibiotics in both human and veterinary medicine, their use as growth promoters in animal husbandry and as prophylactics in agriculture, have created a selective pressure leading to the emergence and spread of antibiotic resistances (AR) (Rodríguez-Rojas et al., 2013). While pathogens and opportunistic species represent a direct threat to human and animal health when carrying AR due to their difficult eradication, resistant non-pathogenic commensal species constitute an indirect hazard, as the major concern is the horizontal transfer of AR determinants to pathogens (Devirgiliis et al., 2013).

Leuconostoc spp. are lactic acid bacteria (LAB) frequently involved in the manufacture and ripening of fermented foods, where they contribute to the organoleptic and rheological properties of the final product. Very limited information on the antimicrobial susceptibility profiles of *Leuconostoc* spp. exits, and scarce data are available on the AR determinants already spread among food-borne strains, as well as the genetic organization of AR genes.

In this study, whole genome analysis was used to assess the genetic basis of atypical AR displayed by three *Leuconostoc mesenteroides* strains (LBE15, LBE16 and LBT16) and its transferability potential.

Material and Methods

Antibiotic susceptibility testing. The minimum inhibitory concentration (MIC) of several antibiotics was determined by microdilution using VetMIC plates for LAB.

Amplification of AR determinants. Total and plasmid DNA were extracted using the Wizard Genomic DNA purification kit (Promega) and the method of O'Sullivan and Klaenhammer (1993), respectively. Specific primers were used to amplify different AR genes by PCR (Rizzotti et al., 2005).

Sequencing and bioinformatics analysis. Whole-genome sequencing was performed using the Illumina HiSeq2000 platform with a paired-end library. Quality of reads was verified using FastQC software, and *de novo* assembly was performed with the SPAdes Assembler version 3.5.0 (Bankevich et al., 2012). The genome sequences of the three *Leuconostoc* strains were annotated by the NCBI Prokaryotic Genome Annotation Pipeline (Campedelli et al., 2015). The plasmidome analysis of the *Leuconostoc* strains was accomplished with PLACNET software (Lanza, et al., 2014) and involved the following steps: assembly, scaffold links (Bowtie2), plasmid protein detection and reference search.

Location of AR genes by DNA hybridization. Total and plasmid DNA of *L. mesenteroides* LBE15 and LBE16 were independently hybridized with internal segments of *erm*(B) and *tet*(S) genes.

Results and Discussion

The MICs of ampicillin, ciprofloxacin, clindamycin, chloramphenicol, erythromycin, gentamicin, kanamycin, linezolid, neomycin, penicillin, rifampicin, streptomycin, tetracycline, trimethoprim, vancomycin, and virginiamycin were determined for *L. mesenteroides* LBE15, LBE16 and LBT16 strains. LBE15 showed atypical resistance to erythromycin and clindamycin. LBE16 proved to be resistant to tetracycline (MIC>32 µg/ml), kanamycin (MIC=512 µg/ml), neomycin (MIC=32 µg/ml), streptomycin (MIC=128 µg/ml) and virginiamycin (MIC=128 µg/ml). A moderate resistance to tetracycline was detected in LBT16 strain (MIC=16 µg/ml).

PCR with specific primers gave positive amplification for *erm*(B) in LBE15 and for *tet*(S) in LBE16. No amplification was obtained for *tet*(K), *tet*(L), *tet*(M), *tet*(O), *tet*(S), and *tet*(W) when DNA from strain LBT16 as a template was used.

Genome analysis confirmed the presence of *erm*(B) and *tet*(S), in LBE15 and LBE16, respectively (Fig. 1). In the surrounding region of *erm*(B), *orf*s encoding plasmid-replication proteins and two genes encoding proteins involved in mobilization were identified (Fig. 1A), suggesting association of this gene with a plasmid. The *tet*(S) gene in LBE16 (Fig. 1B) was identified in a contig of 171,788 bp, supporting its location in the bacterial chromosome ; this was demonstrated further on by DNA hybridization. In this last strain, two other contigs encoding AR genes were identified (Fig. 1B). One of the contigs showed a cluster of genes encoding aminoglycoside resistance: *aadE*, *sat4*, *aphA-3*, and *mmr*. Further, a small contig capable of encoding two *orf*s, of which one showed extensive homology to *vat*(E) was identified.

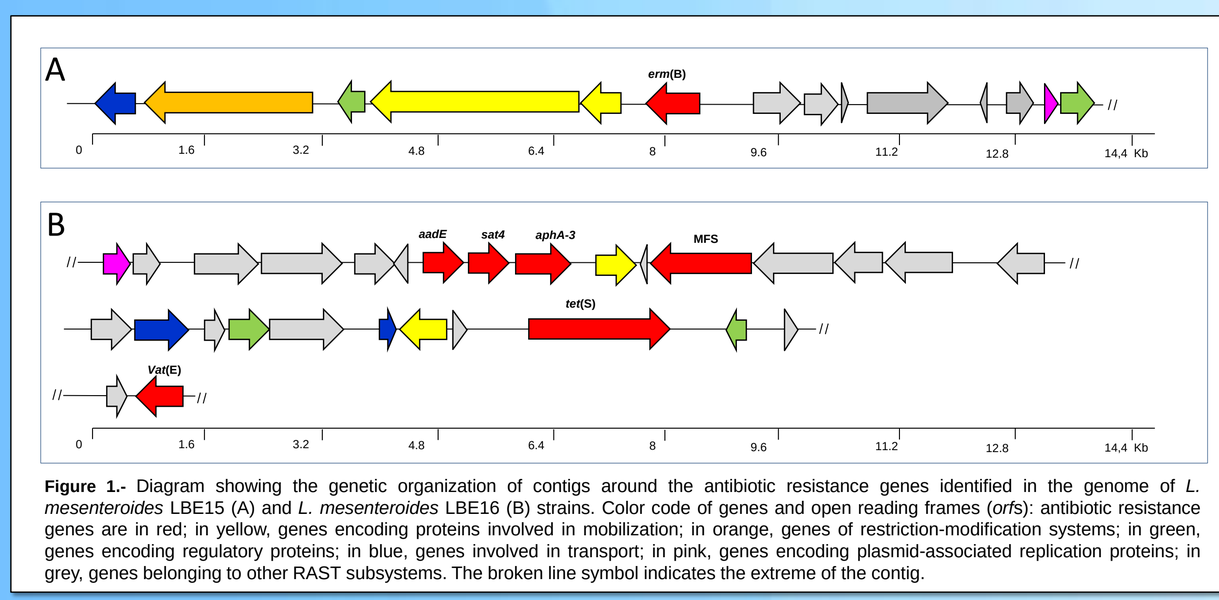

Figure 1.- Diagram showing the genetic organization of contigs around the antibiotic resistance genes identified in the genome of *L. mesenteroides* LBE15 (A) and *L. mesenteroides* LBE16 (B) strains. Color code of genes and open reading frames (*orf*s): antibiotic resistance genes are in red; in yellow, genes encoding proteins involved in mobilization; in orange, genes of restriction-modification systems; in green, genes encoding regulatory proteins; in blue, genes involved in transport; in pink, genes encoding plasmid-associated replication proteins; in grey, genes belonging to other RAST subsystems. The broken line symbol indicates the extreme of the contig.

These genes were not searched for by conventional PCR because their presence is uncommon in LAB. However, they may explain the atypical resistances of LBE16 to aminoglycosides and virginiamycin. Upstream of the *aadE-sat4-aphA-3* cluster, an *orf* that could encode a plasmid-replication protein was detected, indicating again a plasmid location.

Dedicated bioinformatics software, such as PLACNET, allows the *in silico* characterization of the bacterial plasmidome (Fig. 2). This analysis may be helpful to estimate the risk of transfer, as location on mobile genetic elements would certainly increase the transference capability of a any gene. Association of *erm*(B) in LBE15 with a plasmid was demonstrated by hybridization. The plasmid could be transferred to *Enterococcus* spp. under laboratory and cheese conditions (Flórez et al., unpublished).

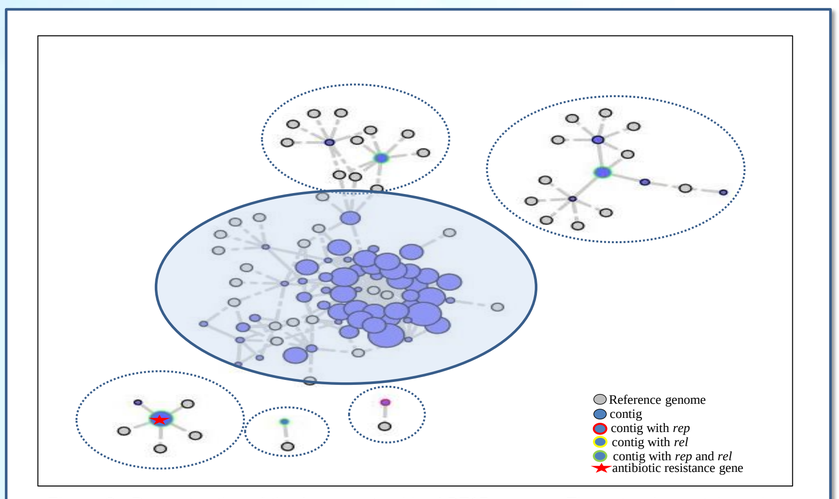

Figure 2.- Reconstruction of the *L. mesenteroides* LBE15 genome. The networks contain nodes of two different colors (blue for contigs, grey for reference genomes). The size of reference nodes is always the same. The size of the contig nodes is proportional to the contig length. Besides, outlines are red for contigs containing RIP proteins, yellow for relaxases and green for both proteins. Edges are either solid (scaffold links) of dotted (homologous references).

Surprisingly, genome analysis of *L. mesenteroides* LBT16 did not revealed the presence of any known tetracycline resistance gene. Close inspection of dubious genes in the LBT16 genome categorized as tetracycline-resistance genes were not considered true AR determinants, based on gene content and gene context: they were chromosomally encoded and present in many LAB strains susceptible to this antibiotic. The moderate resistance of LBT16 to tetracycline (two dilutions higher than EFSA's cut-off) might be due to the activity of a new, dedicated, non-yet characterized mechanism but, most probably, to the contribution of one or more intrinsic mechanisms (cell wall impermeability, (over)expression of unspecific efflux systems, mutations in indigenous genes, ribosome configuration, etc.).

Conclusions

1.- As concerns AR, genome sequencing and bioinformatics analyses are considered affordable tools for the safety assessment of food bacteria.
2.- These techniques allow full characterization of well-known AR genes beyond PCR-based approaches, identifying in most cases structures, such as plasmids, transposons, insertion elements, etc., that may contribute to AR spread; this can help to assess the actual risk of AR genes.
3.- However, sequencing and bioinformatics techniques alone do not eliminate the concerns when AR genes are not identified in strains showing atypical resistances. Further research on the subjacent resistant mechanisms will be needed to assure the safety of these strains in the food chain.

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