Ann Microbiol (2015) 65:833–839
DOI 10.1007/s13213-014-0924-x

ORIGINAL ARTICLE

Hydrogen peroxide-mediated killing of *Caenorhabditis elegans* by *Enterococcus italicus* and *Lactococcus garvieae* isolated from food

Francesca Borgo · Francesco Ballestriero · Chiara Ferrario · Maria Grazia Fortina

Received: 3 April 2014 / Accepted: 28 May 2014 / Published online: 20 June 2014

Abstract In this study, we used the nematode *Caenorhabditis elegans* as a model to assess the pathogenic potential of two species isolated from food, *Enterococcus italicus* and *Lactococcus garvieae*, for which few indications on pathogenicity are available. We identified the conditions under which *E. italicus* and *L. garvieae* are able to kill the nematode and suggest that the production of hydrogen peroxide (H_2O_2) by these two bacteria was involved in the death of *C. elegans* in our model system. The efficacy of *E. italicus* and *L. garvieae* to kill *C. elegans* differed, most likely related to each species' distinct ability to accumulate H_2O_2 (4.9 mM and 0.9–1.1 mM, respectively). Genome analysis of both species revealed that the genome of *E. italicus* contains a gene encoding a NADH oxidase which shows high amino acidic similarity with H_2O_2-forming NOX-1 enzymes, while that of *L. garvieae* contains a gene codifying for a water-forming NADH-oxidase (NOX-2). Reverse transcriptase-PCR experiments carried out in presence of flavin adenine dinucleotide (50 mM) confirmed the presence of the two different genes and likely explains the different toxicity of *E. italicus* and *L. garvieae* against *C. elegans* in our study. The results obtained show for the first time the production of H_2O_2 in *E. italicus* and *L. garvieae* and indicate its toxic effect in the nematode *C. elegans*.

Keywords *Caenorhabditis elegans* · *Enterococcus italicus* · *Lactococcus garvieae* · Emerging foodborne pathogens · Hydrogen peroxide · *nox* genes

Introduction

In the last two decades, foodborne diseases have emerged as an important and growing public health concern (EFSA 2008). This has led to the development and use of methods to monitor and detect undesirable microorganisms in food products. Today, together with conventional microbiological analyses, more sensitive methods, such as immunology-based methods involving antigen–antibody interactions and PCR analysis for DNA characterization, are available. However, little is known of the presence and function of virulence factors in several emerging foodborne bacteria or of their impact bacteria on specific ecosystems, and their mechanism of pathogenesis is not fully understood (Hatcher et al. 2012). Innovative strategies and methods are thus needed for surveying foodborne disease and food contamination (Tauxe 2002).

The nematode *Caenorhabditis elegans* can be used as a simple and well-established model host in studies on emerging foodborne pathogens. Comprehensive studies have reported that this worm is a versatile model metazoan in which to assess the virulence of many human pathogens (Ewbank and Zugasti 2011). This free-living nematode offers numerous practical and economic advantages, including its small size (adults are approx. 1 mm long) and short life cycle (only 3 days from the fertilized egg to a sexually active adult) (Blaxter 2011). Moreover, the bacteriovorous *C. elegans* can be easily challenged with pathogens, and its life span is readily measured using simple assays (Tan et al. 1999; Ballestriero et al. 2010; Penesyan et al. 2013). Laboratory experiments have shown that *C. elegans* responds differently to pathogenic species, such as *Salmonella enterica* and *Enterococcus*

Francesca Borgo and Francesco Ballestriero contributed equally to this work.

F. Borgo (✉) · C. Ferrario · M. G. Fortina
Division of Food Microbiology and Bioprocesses, Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, 2 Celoria, 20133 Milan, Italy
e-mail: francesca.borgo@unimi.it

F. Ballestriero
School of Biotechnology and Biomolecular Sciences and Centre for Marine Bio-Innovation, University of New South Wales, Sydney, NSW, Australia

faecalis (Aballay et al. 2000; Garsin et al. 2001), and to probiotic bacteria, such as *Lactobacillus paracasei* and *Bifidobacterium* spp. (Lee et al. 2011). *Caenorhabditis elegans* also recognizes toxic solutes, such as alcohol, reactive oxygen species, heavy metals and sulphydryl reactive compounds, which induce the production of cellular stress proteins by inhibiting feeding (Sifri et al. 2002). Consequently, worm-based assays have been widely employed to study two different types of mechanisms of pathogenesis. Bacterial pathogens can kill the nematode either through an infection-like process (Tan et al. 1999) or by diffusible toxins (Jansen et al. 2002; Moy et al. 2004). In the infection model system, *C. elegans* ingests the bacteria and dies over the course of several days, depending on the pathogen. In contrast, killing by diffusible toxins occurs more quickly and does not require contact with viable bacterial cells.

The aim of our study was to employ the *C. elegans* system to assay the pathogenic potential of two emerging foodborne bacteria, *Enterococcus italicus* and *Lactococcus garvieae*. The genus *Enterococcus* encompasses a considerable number of different species, some of which are well characterized in regards to their pathogenicity (Eaton and Gasson 2001; Semedo et al. 2003). *Enterococcus italicus*, which is dairy in origin (Fortina et al. 2004), has been found to have a low virulence profile (Fortina et al. 2008; Borgo et al. 2009), but the identification of a clinical isolate belonging to *E. italicus* (Carvalho et al. 2008) and genome analysis of the type strain of the species (Borgo et al. 2013) indicate the possible presence of several virulence factors for which further studies are needed. *Lactococcus garvieae* is a pathogen that causes septicaemia in fish and serious economic damage to fish aquaculture worldwide (Vendrell et al. 2006). In addition, this bacterium has been found in different food matrices (Fortina et al. 2003; Fernández et al. 2010; Ferrario et al. 2012), and several cases of *L. garvieae* infection in humans have been reported (Fihman et al. 2006; Aubin et al. 2011; Russo et al. 2012). However, despite the growing importance of *L. garvieae* in both human and veterinary medicine, little data directly related to pathogenicity factors are available. In our study, we show that *E. italicus* and *L. garvieae* are able to produce hydrogen peroxide (H_2O_2) and that the production of H_2O_2 is likely related to their potential pathogenicity.

Materials and methods

Bacterial and nematode strains

Bacterial strains were grown on Brain Heart Infusion (BHI medium (Difco, Necton Dickinson, Sparks, MD) at 37 °C. Liquid cultures were typically grown without shaking. We used *Enterococcus italicus* strain TP1.5^T (DSM 15952^T) isolated from Italian cheese and *Lactococcus garvieae* strains TB25 and LG9, originating from Italian cheese and diseased fish, respectively. *Caenorhabditis elegans* strain Bristol N2 (Brenner 1974) was maintained by using standard practices (Sulston and Hodgkin 1988).

Nematode killing assay

Bacterial cultures were grown in BHI medium at 37 °C and diluted to an optical density at 600 nm (OD_{600}) of 0.1–0.2 in fresh medium; 10-μl aliquots were then spread onto 35-mm BHI agar plates and the plates incubated overnight at 37 °C. AnaeroGen (Oxoid, Basingstoke, UK) envelopes and jar containers were used to generate an anaerobic environment. Before the nematodes were transferred to the anaerobically grown lawns of *E. italicus* and *L. garvieae*, the plates were cooled aerobically at room temperature for 30–60 min. Nematodes at the L4 developmental stage were suspended and washed twice in M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$, H_2O to 1 l), then transferred to the bacterial lawns in drops (5–10 ml; 30–40 per plate) and incubated at 25 °C. Worms were scored for survival every 24 h. Nematodes that did not respond to touch with a platinum wire pick were considered to be dead. For the experiments with catalase (Sigma, St. Louis, MO), catalase was mixed into liquid BHI agar at a concentration of 1,000 U ml^{-1}. Assays for each bacterial strain and condition were carried out at least twice, and each experiment was done in triplicate.

H_2O_2 sensitivity assays

Bacteria were grown in BHI medium at 37 °C until the mid-log phase (OD_{600}=0.4–0.6) was reached, centrifuged for 2 min at 10,000 *g* and 4 °C, washed in ice-cold saline solution (0.9 % NaCl, w/v) and resuspended in fresh BHI medium. Resuspended bacteria were added to microtiter plate wells in duplicate containing twofold dilutions of H_2O_2 (Sigma,) in BHI medium and incubated for 30 min. Aliquots from each well were applied to BHI agar plates containing 200 U of catalase (Sigma) per milliliter for viable counts. The concentration of H_2O_2 required to cause a 99.9 % decrease in the number of colonies compared to the negative control without H_2O_2 was recorded as the minimum bactericidal concentration (MBC).

H_2O_2 measurement

To measure H_2O_2 accumulation upon aeration of cultures grown under anaerobic conditions, we cultivated 100-ml BHI medium cultures of *E. italicus* and *L. garvieae* strains in 500-ml flasks at 37 °C without agitation in jar containers. Cultures were removed from the anaerobic conditions and aerated on an orbital shaker. To measure bacterial growth and H_2O_2 accumulation in aerobic cultures, overnight cultures

were grown without shaking, diluted into 100 ml of prewarmed BHI medium aerated at 37 °C and centrifuged at 10,000 *g*. The H_2O_2 levels of bacterium-free culture eluates were measured with horseradish peroxidase type VI-A (Sigma) at 505 nm. The H_2O_2 was quantified using standard curve determined with H_2O_2 (Sigma) solutions at concentrations ranging from 1.5 to 100 mmol l^{-1}.

Catalase and NADH oxidase activity assay

Aerobic cultures grown under anaerobic conditions were harvested at an OD_{600nm} of 0.4–0.6 and the cells washed with ice-cold phosphate buffer (PB) (50 mmol l^{-1} KH_2PO_4, 0.5 mmol l^{-1} EDTA, pH 7.0). Cells were disrupted using glass beads (diameter<106 μm; Sigma) and a Bead Beater (Precellys®24; Bertin Technologies, Montigny le Bretonneux, France) and were then centrifuged for 15 min at 4 °C at 10,000 *g*. The protein concentration was determined by the Bradford (1976) method using serum albumin as a standard.

Catalase activity was measured by adding 25 ml of cell extract to a cuvette containing 0.1 % H_2O_2 in 1 ml of 50 mmol l^{-1} PB. The rate of H_2O_2 decomposition was recorded as the change in adsorption at 240 nm. The extinction coefficient for H_2O_2 (ε_{240}=0.0436 cm^2 $mmol^{-1}$) was used to calculate catalase activity units. One unit decomposes 1 mmol H_2O_2 min^{-1}.

NADH oxidase activity was measured by adding 25 ml of cell extract to 1 ml reaction mixture containing 150 mmol l^{-1} NADH (Sigma) with or without 50 mmol l^{-1} flavin adenine dinucleotide (FAD; Sigma) in the PB, and absorbance at 340 nm was monitored for 2 or 3 min.

nox detection

Total DNA was extracted as described in Borgo et al. (2013). The NADH oxidase gene (*nox*) primers were designed on the basis of comparisons of the nucleotide sequences of the *E. italicus* DSM 15952^T and *L. garvieae* TB25 and LG9 whole genome shotgun sequence (Accession nos: PRJNA61487, AGQX01000000, AGQY01000000, respectively). Before use, the sequence of the forward primer was checked with BLAST (Basic Local Alignment Search Tool) at the National Center for Biotechnology Information (NCBI) to ensure that no matches with other genes were present (Altschul et al. 1990). The primers were synthesized by PrimmBiotech (Milan, Italy), and the sequences were 5′-GCATTATATGTCGGCGGTGT-3′ and 5′-CGCAATAATTGGCCATGAGC-3′ (forward and reverse, respectively). Each 25-μl reaction mixture contained 100 ng of bacterial DNA, 2.5 μl of 10× reaction buffer, 200 mM of a deoxynucleoside triphosphate mixture, 2.5 mmol l^{-1} $MgCl_2$, 0.5 mmol l^{-1} of each primer and 0.5 U of *Taq* polymerase (Dream *Taq* DNA-polymerase; MBI-Fermentas, Vilnius, Lithuania). Amplification was performed in a Gene Amp PCR System 2400 Thermal Cycler (Perkin-Elmer, Norwalk, CT) and consisted of 30 cycles of 94 °C for 1 min (denaturation), 58 °C for 1 min (annealing) and 72 °C for 2 min (extension), followed by an additional 7-min elongation period at 72 °C. PCR products were separated on 1.5 % agarose gel, stained with ethidium bromide in 1× Tris–acetate–EDTA buffer (40 mmol l^{-1} Tris-acetate, 1 mmol l^{-1} EDTA, pH 8.0) and photographed under UV light.

Quantitative RT-PCR

RNA was extracted with a NucleoSpin RNA II extraction kit (Macherey-Nagel, GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. The concentration and purity of the extracted mRNA were optically determined using a UV–Vis spectrophotometer (SmartSpecTM Plus; Bio-Rad, Hercules, CA), which measured the absorbance of the sample at wavelengths of 260 and 280 nm. Based upon the final concentrations, 100 ng of mRNA was rewritten into cDNA using a RevertAid First Strand cDNA Synthesis kit (MBI-Fermentas) and thermal cycling of 5 min at 25 °C, 30 min at 42 °C and 5 min at 85 °C. The mRNA expression levels of *nox* were analyzed with SYBR® Green technology (Life Technologies, Carlsbad, CA) in real-time quantitative PCR (qPCR) using the SsoFast EvaGreen Supermix (Bio-Rad) on a Bio-Rad CFX96 system according to the manufacturer's instructions. In order to quantitatively measure the expression of the *E. italicus* and *L. garvieae nox* genes, we used the housekeeping gene 16S rRNA as internal control to which all other gene expression was normalized. The primers used were 5′-GAAGTCGTAACAAGG-3′ and 5′-GTGTCT CAGTCCAGTGTGG-3′ for 16S rRNA and the previously cited for *nox* genes. Samples were examined for differences in gene expression using relative quantification in which relevant gene expression is normalized to a housekeeping gene, according to Pfaffi (2001).

The specificity of the primers was tested with melting curves during amplification and in 1 % agarose gels. qPCR amplification was carried out as follows: an initial hold at 96 °C for 30 s, followed by 40 cycles at 96 °C for 2 s and at 60 °C for 5 s. The amount of template cDNA used for each sample was 1.25 ng.

Results

E. italicus- and *L. garvieae*-mediated killing of *C. elegans*

Preliminary experiments were carried out to verify their ability to grow in BHI medium under aerobic or anaerobic conditions. No significant differences in cell growth were found

when the bacterial strains were grown under aerobic or anaerobic conditions (Table 1). The dry cell weight under aerobic or anaerobic conditions was also similar after the addition of FAD (50 mmol l^{-1}). Following growth under aerobic conditions *E. italicus* and *L. garvieae* did not have any apparent deleterious effect on the nematode *C. elegans* when the bacteria were provided to the worms as the sole source of food (Fig. 1a). However, when grown under anaerobic conditions on BHI medium and then exposed to aerobic conditions, the *E. italicus* and *L. garvieae* strains tested rapidly killed *C. elegans* (Fig. 1b). Furthermore, nematode mobility decreased within 45 min (data not shown). *Lactococcus garvieae* strains TB25 and LG9 killed the nematode within 5 days, while in the presence of *E. italicus* TP1.5 ^{T} 50 % of the nematodes died within 2–4 h, and nearly all of the nematodes had died by 8 h (expanded view ofFig. 1b). As shown in the expanded view of Fig. 1b, the addition of exogenous catalase to the solid BHI medium allowed the nematodes to remain mobile, with 100 % of survival. The same behavior was observed when *L. garvieae* strains were tested.

Killing of *C. elegans* is related to H_2O_2 production

On the basis of previously published data (Moy et al. 2004), we examined the capacity of the *E. italicus* and *L. garvieae* strains tested to produce H_2O_2. *E. italicus* TP1.5 and *L. garvieae* TB25 and LG9 accumulated H_2O_2 in relation to cell growth, with the concentration of H_2O_2 in the culture media of these strains reaching 4.9, 0.9 and 1.1 mmol l^{-1}, respectively, within the first 8 h of incubation (Table 1). The ability to accumulate H_2O_2 was related to a high level of resistance to this toxic substance. When cells were exposed to 0.1 and 1 mmol l^{-1} of H_2O_2 for 30 min, the survival of the tested strains was unaffected. Following exposure to 10.0 mmol l^{-1} H_2O_2, *E. italicus* decreased to undetectable levels (0.5 log), whereas the number of *L. garvieae* cells decreased approximately by 2 log cycles. These results indicate that *E. italicus* TP1.5 has a higher ability to survive in the presence of exogenously added H_2O_2 than the *L. garvieae* strains tested.

Detection of NADH oxidase genes and expression studies

To study the biosynthetic enzymes involved in H_2O_2 production we investigated the genomes of all three strains (for accession numbers, see section *nox* detection) for the presence of genes related to this process. We did not detect any genes encoding catalase and NADH peroxidase enzymes, but in both species we did find a nucleotide sequence highly similar to that of *nox* genes which encoded NADH oxidase activity. Thus, *E. italicus* and *L. garvieae* would appear to use oxidases to reduce molecular oxygen to form H_2O_2, and the absence of NADH peroxidase, the major mechanism to scavenge H_2O_2, could result in increased accumulation of this reactive molecule. Two types of NADH oxidases are known, corresponding to H_2O_2-forming oxidase (NOX-1) and H_2O-forming oxidase (NOX-2). A comparison between the amino acid sequences of NOX in the tested strains and other reported amino acid sequences in other species and genera showed a high similarity (78 %) of the *nox* gene of *E. italicus* with NOX-1 enzymes and a lower level of similarity with the water-forming NADH oxidases (NOX-2). On the contrary, the NADH oxidase found in the *L. garvieae* strains showed a higher similarity (81 %) with NOX-2 enzymes.

Figure 2 shows the alignment based on the primary structure of the *E. italicus* 446-amino acid NOX protein and

Table 1 Effects of different culture conditions on dry cell weight, NADH oxidase activity and hydrogen peroxide accumulation in BHI medium at 25 °C after a 24-h incubation with *Enterococcus italicus* strain TP1.5^{T} and *Lactococcus garvieae* strains TB25 and LG9

Bacterial strain	Growth conditions	DCW±SD (mg l^{-1})	NADH oxidase activity (U mg^{-1})	Hydrogen peroxide (mmol l^{-1})			
				2 h	4 h	6 h	8 h
Enterococcus italicus strain TP1.5^{T}	Anaerobic	131±7	0	0	0	0	0
	Aerobic	135±6.8	10.3±0.5	1.2±0.1	2.1±0.2	3.5±0.3	4.9±0.4
	Aerobic+FAD[a]	129±6.5	62.1±3.1	np	np	np	np
Lactococcus garvieae strain TB25	Anaerobic	142±7.1	0	0	0	0	0
	Aerobic	148±7.4	7.1±0.4	0.3±0.0	0.5±0.0	0.7±0.0	0.9±0.0
	Aerobic+FAD[a]	139±7	7.3±0.4	0.3±0.0	0.4±0.0	0.6±0.0	0.9±0.0
Lactococcus garvieae strain LG9	Anaerobic	167±8.4	0	0	0	0	0
	Aerobic	171±8.5	6.1±0.3	0.3±0.0	0.6±0.0	0.9±0.0	1.1±0.1
	Aerobic+FAD[a]	163±8.2	6.4±0.3	0.3±0.0	0.4±0.0	0.6±0.0	0.9±0.1

Values are presented as the mean ± standard deviation (SD)

DCW Dry cell weight, *np* not performed

[a] 50 mmol l^{-1} flavin adenine dinucleotide (FAD)

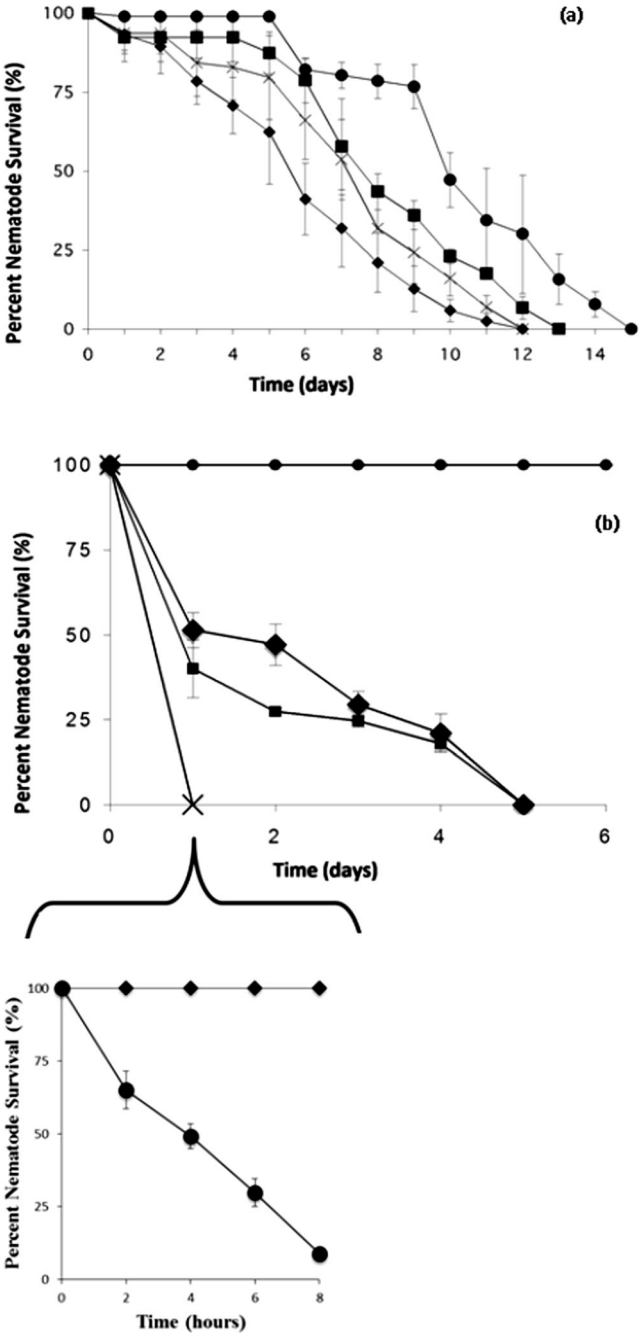

Fig. 1 *Caenorhabditis elegans* survival when fed *Enterococcus italicus* strain TP 1.5 and *Lactococcus garvieae* strains TB25 and LG9, respectively. The tested strains were grown on solid BHI medium under aerobic conditions (**a**) and under anaerobic followed by aerobic conditions (**b**). L4-stage *C. elegans* was transferred onto the lawns of tested strains, incubated at 25 °C and scored for survival. *Symbols*: *Filled black circle Escherichia coli* strain OP50 (positive control), *filled square L. garvieae* strain TB 25, *X E. italicus* strain TP 1.5, *filled diamond L. garvieae* strain LG9. *Expanded view C. elegans* survival, expressed in hours, when the worm was fed *E. italicus* grown under anaerobic/aerobic conditions in the presence (*filled diamond*) or absence (*filled circle*) of exogenous catalase

L. garvieae strains TB25 and LG9 462 amino acid NOX proteins. BLAST analysis of the amino acid sequence in NOX revealed conserved sequences, including the known FAD and NADH binding domain motifs and the LAXXAXXXG sequence, which appears to be highly conserved in flavoproteins. A cysteine residue in position 42 characterizing water-forming NADH oxidase (NOX-2) was found only in the *L. garvieae* NADH oxidase gene (Fig. 2): this amino acid allows the capture of H_2O_2, forming the sulfenic acid (cys42-SOH) intermediate. Electrons from a second molecule of NADH reduce sulfenic acid to water and thiolate (Jiang et al. 2005). Based on our results, *E. italicus* appears to possess a NADH oxidase H_2O_2-forming (NOX-1) in the presence of FAD, as also verified by measuring the enzymatic activity and the relative gene expression. Our results are similar to those previously published in which the addition of free FAD stimulated the oxidase activity of NOX-1 (Riebel et al. 2002), but not the activity of the NOX-2 enzyme (Jiang et al. 2005), thereby verifying the enzymatic activity in the presence/absence of FAD. As shown in Table 1, FAD added exogenously to the culture system at 50 mmol l^{-1} increased the activity of NADH oxidase in *E. italicus* (by 6-fold), but did not significantly change the activity of this enzyme in the two *L. garvieae* strains tested.

To confirm the nature of the NOX enzymes found in *E. italicus* and *L. garvieae*, we also performed qRT-PCR experiments, designing a primer set able to amplify a 222-bp internal region of the *nox* genes. Expression levels under standard culture conditions (BHI medium, 37 °C, 16 h of incubation, anaerobic conditions) and under aerobic conditions (BHI medium, 37 °C, 16 h of incubation) in the presence or absence of exogenously added 50 mmol l^{-1} FAD. In the latter case, a ten-fold increase in the expression level of *nox* was observed only in the *E. italicus* strain, confirming that in this species the NADH oxidase gene encodes a putative H_2O_2-forming oxidase, whose expression is positively modulated in presence of additional FAD.

Discussion

In our study, we used *C. elegans* as an innovative tool for studying two emerging foodborne pathogens, *E. italicus* and *L. garvieae*, for which limited information on their potential pathogenicity is available. This study has not fully resolved the question of the nature of the killing of *C. elegans* by *E. italicus* and *L. garvieae*, but our results do support the hypothesis of a mechanism involving H_2O_2, which has been proposed for other enterococcal species (Garsin et al. 2001; Moy et al. 2004). The first step in our study was to grow the three strains to be tested under different culture conditions. This assay revealed that *E. italicus* and *L. garvieae* displayed a killing phenotype when grown under anaerobic conditions,

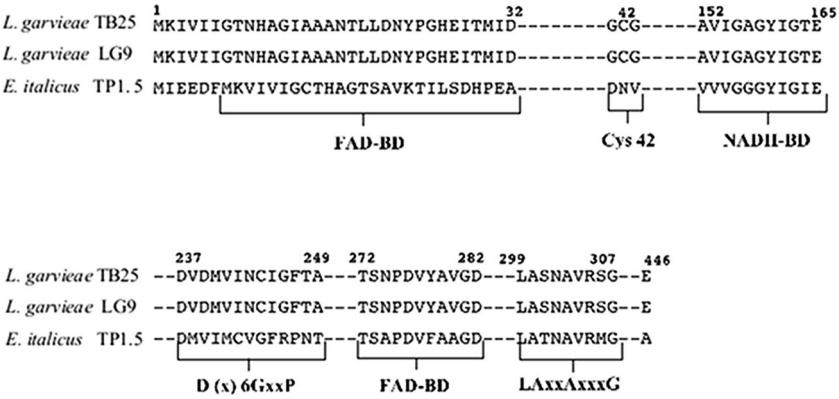

Fig. 2 Structure-based amino acid alignment of NADH oxidase in *L. garvieae* TB25, *L. garvieae* LG9 and *E. italicus* TP 1.5. *Shading* Conserved sequence motifs. *BD* Binding domain, *FAD* flavin adenine dinucleotide

leading us to hypothesize that the tested strains were producing a toxic compound during the anaerobic growth phase. However, further experiments revealed that the toxin H_2O_2 was not produced until the lawns of *E. italicus* and *L. garvieae* were exposed to oxygen. This led to a new hypothesis, namely, that following exposure to oxygen, these bacteria accumulate the H_2O_2 produced because the absence of NADH peroxidase in the cells (as verified in the genomic analysis of the strains) cannot adequately scavenge H_2O_2. The bacterial strains tested showed a different ability to kill *C. elegans* which was linked to a distinct accumulation of H_2O_2 (4.9 mmol l^{-1} for *E. italicus* and 0.9–1.1 mmol l^{-1} for *L. garvieae*). Furthermore, nematode killing was prevented by an addition of catalase to cultures of *E. italicus* and *L. garvieae* that had killed the worms.

This difference in the ability to kill *C. elegans* can be explained by the results of our analysis of the amino acid sequence of the NADH oxidases found in the two species. A cysteine residue in position 42, present only in the *L. garvieae* NADH oxidase gene (Fig. 2) characterizes H_2O-forming oxidase (nox-2); this amino acid allows the capture of H_2O_2, forming the sulfenic acid (cys42-SOH) intermediate. Electrons from a second molecule of NADH reduce sulfenic acid to water and thiolate (Jiang et al. 2005). *Enterococcus italicus* seems to possess a NADH oxidase that forms H_2O_2 (nox-1), as also verified by measuring the enzymatic activity and the relative gene expression in the presence of FAD. Riebel et al. (2002) reported that the addition of this cofactor induces an increase expression of *nox*1 gene, as found in our study of the *E. italicus* NADH oxidase.

Here, we report for the first time genotypic and phenotypic evidence of a possible mechanism of virulence in two potential emerging food pathogens, *E. italicus* and *L. garvieae*. H_2O_2 production was reported and its toxic effects studied in vivo. *Caenorhabditis elegans*, a free-living nematode, provided the model for investigating toxin-mediated killing. The presence along the genome of *E. italicus* NOX-1 and *L. garvieae* NOX-2 could explain the distinct killing phenotype of these two species against *C. elegans*. This genetic diversity may also explain the different accumulation of H_2O_2 and, consequently, the distinct killing phenotype of *E. italicus* and *L. garvieae* against *C. elegans*. As reported for other Gram-positive bacteria, including pathogens, such as *Streptococcus pneumoniae* and *S. pyogenes* (Spellerberg et al. 1996; Jansen et al. 2002), the H_2O_2 produced by the cells can contribute to virulence by damaging host tissue or surrounding cells. Further studies are necessary to understand if this molecule may play a role in *E. italicus* and *L. garvieae* colonization or infection and if additional virulence factors are responsible for nematode killing.

In conclusion, in this preliminary study we reported for the first time genotypic and phenotypic evidence of a possible mechanism of virulence in two potential emerging food pathogens. The results of our study also highlight the utility of the *C. elegans* pathogenicity model for identifying bacterial virulence factors.

References

Aballay A, Yorgey P, Ausubel FM (2000) *Salmonella typhimurium* proliferates and establishes a persistent infection in the intestine of *Caenorhabditis elegans*. Curr Biol 10:1539–1542

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Aubin GG, Bémer P, Guillouzouic A, Crémet L, Touchais S, Fraquet N, Boutoille D, Reynaud A, Lepelletier D, Corvec S (2011) First report of a hip prosthetic and joint infection caused by *Lactococcus garvieae* in a woman fishmonger. J Clin Microbiol 49:2074–2076

Ballestriero F, Thomas T, Burke C, Egan S, Kjelleberg S (2010) Identification of compounds with bioactivity against the nematode *Caenorhabditis elegans* by a screen based on the functional genomics of the marine bacterium *Pseudoalteromonas tunicata* D2. Appl Environ Microbiol 76:5710–5717

Blaxter M (2011) Nematodes: the worm and its relatives. PLoS Biol 9: e1001050

Borgo F, Ricci G, Arends K, Schiwon K, Grohmann E, Fortina MG (2009) Evaluation of plasmid content and tetracycline resistance conjugative transfer in *Enterococcus italicus* strains of dairy origin. Curr Microbiol 59:261–266

Borgo F, Ferrario C, Ricci G, Fortina MG (2013) Genotypic intraspecies heterogeneity of *Enterococcus italicus*: data from dairy environments. J Basic Microbiol 53:20–28

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Brenner S (1974) The genetics of *Caenorhabditis elegans*. Genetics 77: 71–94

Carvalho Mda GS, Steigerwalt AG, Morey RE, Shewmaker PL, Falsen E, Facklam RR, Teixeira LM (2008) Designation of the provisional new *Enterococcus* species CDC PNS-E2 as *Enterococcus sanguinicola* sp. nov., isolated from human blood, and identification of a strain previously named *Enterococcus* CDC PNS-E1 as *Enterococcus italicus* Fortina, Ricci, Mora, and Manachini 2004. J Clin Microbiol 46:3473–3476

Eaton TJ, Gasson MJ (2001) Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. Appl Environ Microbiol 67:1628–1635

EFSA (European Food Safety Authority) (2008) Scientific opinion of the panel on dietetic products, nutrition and allergies. EFSA J 644:1–44

Ewbank JJ, Zugasti O (2011) *Caenorhabditis elegans*: model host and tool for antimicrobial drug discovery. Dis Model Mech 4:300–304

Fernàndez E, Alegria A, Delgado S, Mayo B (2010) Phenotypic, genetic and technological characterization of *Lactococcus garvieae* strains isolated from a raw milk cheese. Int Dairy J 20:142–148

Ferrario C, Ricci G, Borgo F, Rollando A, Fortina MG (2012) Genetic investigation within *Lactococcus garvieae* revealed two genomic lineages. FEMS Microbiol Lett 332:153–161

Fihman V, Raskine L, Barrou Z, Kiffel C, Riahi J, Berçot B, Sanson-Le Pors MJ (2006) *Lactococcus garvieae* endocarditis: identification by 16S rRNA and *sod*A sequence analysis. J Infect 52:3–6

Fortina MG, Ricci G, Acquati A, Zeppa G, Gandini L, Manachini PL (2003) Genetic characterization of some lactic acid bacteria occurring in an artisanal protected denomination origin (PDO) Italian cheese, the Toma piemontese. Food Microbiol 20:397–404

Fortina MG, Ricci G, Mora D, Manachini PL (2004) Molecular analysis of artisanal Italian cheeses reveals *Enterococcus italicus* sp. nov. Int J Syst Evol Microbiol 54:1717–1721

Fortina MG, Ricci G, Borgo F, Manachini PL, Arends K, Schiwon K, Abajy MY, Gronmann E (2008) A survey on biotechnological potential and safety of the novel *Enterococcus* species of dairy origin, *E. italicus*. Int J Food Microbiol 123:204–211

Garsin DA, Sifri CD, Mylonakis E, Qin KV, Singh KV, Murray BE, Calderwood SB, Ausubel FM (2001) A simple model host for identifying Gram-positive virulence factors. Proc Natl Acad Sci USA 98:10892–10897

Hatcher MJ, Dick JTA, Dunn AM (2012) Disease emergence and invasions. Funct Ecol 26:1275–1287

Jansen WT, Bolm M, Balling R, Chhatwal GS, Schnabel R (2002) Hydrogen peroxide-mediated killing of *Caenorhabditis elegans* by *Streptococcus pyogenes*. Infect Immun 70:5202–5207

Jiang R, Riebel BR, Bommarius AS (2005) Comparison of alkyl hydroperoxide reductase (AhpR) and water-forming NADH oxidase from *Lactococcus lactis* ATCC 19435. Adv Synth Catal 347:1139–1146

Lee J, Yun HS, Cho KW, Oh S, Kim SH, Chun T, Kim B, Whang KY (2011) Evaluation of probiotic characteristics of newly isolated *Lactobacillus* spp.: immune modulation and longevity. Int J Food Microbiol 148:80–86

Moy TI, Mylonakis E, Calderwood SB, Ausubel FM (2004) Cytotoxicity of hydrogen peroxide produced by *Enterococcus faecium*. Infect Immun 72:4512–4520

Penesyan A, Ballestriero F, Daim M, Kjelleberg S, Thomas T, Egan S (2013) Assessing the effectiveness of functional genetic screens for the identification of bioactive metabolites. Mar Drugs 11:40–49

Pfaffi MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:2002–2007

Riebel BR, Gibbs PR, Wellborn WB, Bommarius AS (2002) Cofactor regeneration of NAD^+ from NADH: novel water-forming NADH oxidases. Adv Synth Catal 344:1156–1168

Russo G, Iannetta M, D'Abramo A, Mascellino MT, Pantosti A, Erario L, Tebano G, Oliva A, D'Agostino C, Trinchieri V, Vullo V (2012) *Lactococcus garvieae* endocarditis in a patient with colonic diverticulosis: first case report in Italy and review of the literature. New Microbiol 35:495–501

Semedo T, Santos MA, Lopes MF, Figueiredo Marques JJ, Barreto Crespo MT, Tenreiro R (2003) Virulence factors in food, clinical and reference enterococci: a common trait in the genus? Syst Appl Microbiol 26:13–22

Sifri CD, Mylonakis E, Singh KV, Qin X, Garsin DA, Murray BE, Ausubel FM, Calderwood SB (2002) Virulence effect of *Enterococcus faecalis* protease genes and the quorum-sensing locus *fsr* in *Caenorhabditis elegans* and mice. Infect Immun 70:5647–5650

Spellerberg B, Cundell DR, Sandros J, Pearce BJ, Idänpään-Heikkilä I, Rosenow C, Masure HR (1996) Pyruvate oxidase, as a determinant of virulence in *Streptococcus pneumoniae*. Mol Microbiol 19:803–813

Sulston J, Hodgkin J (1988) The nematode *Caenorhabditis elegans*. Cold Spring Harbor Laboratory, New York

Tan MW, Mahajan-Miklos S, Ausubel FM (1999) Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. Proc Natl Acad Sci USA 96: 715–720

Tauxe RV (2002) Emerging food-borne pathogens. Int J Food Microbiol 78:31–41

Vendrell D, Balcázar JL, Ruiz-Zarzuela I, de Blas I, Girone's O, Muzquiz JL (2006) *Lactococcus garvieae* in fish: a review. Comp Immunol Microbiol Infect Dis 29:177–19