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Structural change in GadD2 of *Listeria monocytogenes* field isolates supports nisin resistance

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ABSTRACT

The lantibiotic nisin is used as a food additive to effectively inactivate a broad spectrum of Gram-positive bacteria such as *Listeria monocytogenes*. In total, 282 *L. monocytogenes* field isolates from German ready-to-eat food products, food-processing environments and patient samples and 39 *Listeria* reference strains were evaluated for their susceptibility to nisin. The MIC_{90} value was < 1500 IU ml^{-1}. Whole genome sequences (WGS) of four nisin susceptible (NS; growth < 200 IU ml^{-1}) and two nisin resistant *L. monocytogenes* field isolates (NR; growth > 1500 IU ml^{-1}) of serotype IIa were analyzed for DNA sequence variants (DSVs) in genes putatively associated with NR and its regulation. WGS of NR differed from NS in the *gadD2* gene encoding for the glutamate decarboxylase system (GAD). Moreover, homology modeling predicted a protein structure of GadD2 in NR that promoted a less pH dependent GAD activity and may therefore be beneficial for nisin resistance. Likewise NR had a significant faster growth rate compared to NS in presence of nisin at pH 7. In conclusion, results contributed to ongoing debate that a genetic shift in GAD supports NR state.

1. Introduction

The foodborne pathogen *Listeria monocytogenes* is widely distributed in the environment and many isolates are able to withstand a broad spectrum of harsh conditions such as low pH (O'Driscoll et al., 1996), low temperatures (Gill and Reichel, 1989) or high salt concentrations (Hwang et al., 2009). The main route of transmission to humans is by consumption of food contaminated with *L. monocytogenes* (Kozak et al., 1996; World Health Organization Food and Agriculture Organization of the United Nations, 2004), which mainly caused sporadic cases and frequently led to outbreaks (Fretz et al., 2010b; Hächler et al., 2013; MacDonald et al., 2005; McIntyre et al., 2015). Raw food products, non-thermally treated, lightly preserved and ready-to-eat (RTE) food generally support growth after contamination with *L. monocytogenes* (Kells and Gilmour, 2004; Kozak et al., 1996; Hächler et al., 2013; Weiler et al., 2013).

Frequently, antimicrobial substances (i.e. herbs, weak organic acids, spices, chelators, bacteriocins, phages) are used as food additives to increase food safety (Gill and Reichel, 1989; Hwang et al., 2009; Joint FAO/WHO Expert Committee on Food Additives, 2010).

For > 50 years, the class I bacteriocin nisin has been commercially used as a food additive often applied to RTE foods by the food industry (Delves-Broughton et al., 1996) and has been granted GRAS (Generally Regarded as Safe) status by the U.S. Food and Drug Administration (Federal Drug Administration, 1988). This 34-residue antibacterial cationic peptide produced by *Lactococcus lactis* subsp. *lactis* and other lactic acid bacteria is active against a wide range of Gram-positive bacteria. Nisin features many sequence variants based on post-translational modifications (Cheigh and Pyun, 2005; Field et al., 2015). Resistance of *L. monocytogenes* to nisin has frequently been reported (Benkerroum and Sandine, 1988; Ferreira and Lund, 1996; Iancu et al., 2012; Katla et al., 2003; Mota-Meira et al., 2000; Rasch and Knøchel, 1998; Ukuku and Shelef, 1997). Reduced susceptibility to nisin is characterized by a complex phenotype and was linked to manifold genotypic characteristics. In spontaneous nisin resistant (NR) variants of *L. monocytogenes* alterations in phospholipid composition and membrane fatty acid composition have been observed (Crandall and Montville, 1998; Davies et al., 1996; Verheul et al., 1997). This evidently results in changes of cell membrane fluidity and may also alter the bacterial cell surface charge (Verheul et al., 1997; Wu et al., 2017),

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which in turn reduces efficiency of nisin binding. In addition, the glutamate decarboxylase system (GAD) was discussed to be relevant for nisin resistance, which comprise three decarboxylases genes *gadD1*, *gadD2*, and *gadD3* that catalyze the conversion of glutamate to γ-aminobutyrate and carbon dioxide. It was discovered that the intracellular ATP levels were reduced in a ΔgadD1 mutant, being only approximately 60% of those of the parent, suggesting that GadD1 contributed significantly to ATP pools and hence supported other mechanisms of NR (Begley et al., 2010; Collins et al., 2011). Moreover, the GAD system contributes to the survival of *L. monocytogenes* at low pH in food environments and in the gastrointestinal tract by reducing intracellular protons. In mild pH conditions GadD1 is required for growth whereas GadD2 is important under extremely acidic pH conditions (Cotter et al., 2005). However, the multifaceted characteristics of nisin resistance are still poorly understood.

In our study, we tested the nisin susceptibility of 282 *L. monocytogenes* field isolates from food, food-processing plants and listeriosis patients as well as 39 *Listeria* reference strains. We focused on isolates derived from milk, cheese and other dairy products, as these isolates have a higher probability of contact with nisin producing bacteria. Using whole genome sequencing (WGS), four nisin susceptible (NS) and two NR *L. monocytogenes* field isolates were compared and analyzed for coding and regulatory sequences potentially involved in nisin resistance. The 3D protein structure of a sequence variant of *gadD2*, which potentially supports reduced susceptibility to nisin, was investigated by a protein modeling approach.

2. Material and methods

2.1. Listeria monocytogenes field isolates and reference strains

A total of 282 *L. monocytogenes* field isolates was sent to the National Reference Laboratory for *L. monocytogenes* at the German Federal Institute of Risk Assessment in Berlin. The bacterial strains were isolated from raw and pasteurized milk, meat, fish, and other food products, as well as food processing plant environments. In addition, *L. monocytogenes* field isolates from human patients in Germany were provided by the Robert-Koch-Institute Wernigerode (Table 1, Supplementary Table S1) (Fretz et al., 2010a, 2010b; Koch and Stark, 2006; Noll et al., 2018; Winter et al., 2009;). Molecular serotypes were determined by PCR according to (Doumith et al., 2004). *Listeria monocytogenes* field isolates were selected according to serotypes most frequently associated with human listeriosis cases, i.e. IIa, IIb and IVb (Allerberger, 2003). In addition, 39 reference strains of the genus *Listeria* were included (Supplementary Table S2). *Listeria* spp. were stored in brain heart infusion (BHI) broth with 15% (v/v) glycerol (Carl Roth GmbH, Karlsruhe, Germany) at −80 °C until use. *Listeria monocytogenes* field isolates were cultured on sheep blood agar (Mast Diagnostika DM 101, Reinfeld, Germany) and were incubated for 24 h at 37 ± 1 °C to check haemolytic activity after −80 °C storage. Haemolytic activity was not altered by storage.

Table 1
Sources and serotypes of *L. monocytogenes* field isolates under study. η indicates the non-linear correlation coefficient. If η reaches one, there is no dispersion within the respective sources or serotype. In contrast, if η is close to zero there is no functional dependence.

Source		IIa	IIb	IIc	IVa	IVb	–	Total
	η	0.769						
Environmental samples	0.761	7	2		1	2		12
Fish and fish products		12	5	3		14		34
Human origin		1		1		5		7
Meat and meat products		18	14	19	2	16		69
Milk/cheese and other dairy products		99	11	2	1	21		134
Other products		2	1	1		7	5	16
Vegetarian food products		2	3	1		4		10
Total		141	36	27	4	69	5	282

2.2. Nisin susceptibility test

One colony was picked from sheep blood agar and bacteria were solubilized in 0.9% (w/v) NaCl and adjusted to 0.5 McFarland standard (VWR International, Ismaning, Germany). Thereafter, bacterial cells were diluted in 1:50 liquid H-medium (Merlin Diagnostika, Bornheim, Germany; Troxler et al., 2000). In addition, a stock solution of nisin (2.5%; Sigma Chemical, Louis, USA) in H-medium containing 3000 IU ml^{-1} was freshly prepared before use. In each well of a 96-well microtiter plate, a total of 0.05 ml of the bacterial suspension and 0.05 ml of H-medium containing nisin in double concentrations, resulting to 0, 5, 10, 15, 20, 50, 75, 100, 150, 200, 400 and 1500 IU ml^{-1} in each well, were shaken for 5 s and incubated for 24 h at 37 ± 1 °C. Subsequently, the microtiter plates were shaken for 5 s, and the OD of each well was measured at a wavelength of 690 nm. Bacterial growth was proven by an $OD_{690nm} > 0.1$ whereas nisin sensitive *Listeria* spp. showed an $OD_{690nm} < 0.1$, provided that the controls including the same *Listeria* spp. without nisin and H-medium with nisin but without bacteria revealed an $OD_{690nm} > 0.1$ and < 0.1, respectively. *Lactococcus lactis* ssp. *cremoris* DSM 20069 was used as an efficacy control for the nisin susceptibility tests. NR was defined as > MIC_{90} of the *L. monocytogenes* field isolates tested in our survey whereas all others were classified as NS. Four NS and two NR were selected for WGS. The field isolates were selected by very low and high MIC and by serotype IIa. To decouple WGS findings from source of isolation, among 134 NS of serotype IIa four field isolates were randomly selected while both NR of serotype IIa were chosen from 7 NR. However, all NR were isolated from milk/cheese and other dairy products (Tables 1 and 2).

Each of these six *L. monocytogenes* field isolates were incubated with and without nisin at sub-inhibitory nisin concentrations (150 IU ml^{-1}) over time in six independent replicates. Each well of a sterile 96-well microtiter plate was inoculated with 0.1 ml of an overnight culture of each of the six *L. monocytogenes* field isolates (approximately 10^5 CFU ml^{-1}). The microtiter plates were centrifuged at 4000 rpm, 4 °C for 10 min and supernatant was discarded. Cell pellets were resuspended in 0.2 ml BHI broth adjusted to pH 7.0 or 5.5 and with 0 or 150 IU ml^{-1} nisin. The microtiter plates were sealed with Breathe-Easy® membrane (Carl Roth) and were incubated at 37 °C. OD was measured daily for 7 days using a FLUOstar OPTIMA microplate reader (BMG Labtech, Ortenberg, Germany).

2.3. DNA preparation and whole genome sequencing

Isolation of genomic DNA (gDNA) was conducted according to manufacturer's procedures using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). A total of 20 ng of gDNA of each *L. monocytogenes* field isolate was subjected to library preparation using the Illumina Nextera® XT DNA sample preparation kit (Illumina, Munich, Germany).

Table 2
Nisin susceptible (NS) and nisin resistant (NR) *L. monocytogenes* serotype IIa field isolates used for whole genome sequencing (WGS) analyses.

Field isolates	MIC [IU ml^{-1}]	Classification	Source (country), year
BfR L41	150	NS	Sewage, food production environment (Germany), 1986
BfR L330	150		Rabbit, DSM20600 (England)
BfR L448	150		Smoked salmon (Germany), 2006
BfR L1079	150		Cheese (Germany), 2010
BfR L245	> 1500	NR	Raw milk (Germany), 1994
BfR L261	> 1500		Raw milk (Germany), 1994

According to manufacturer's instructions, gDNA of each *L. monocytogenes* field isolate was tagged, pooled and paired-end sequenced. Paired-end 300 bp sequencing of the DNA was performed using an Illumina MiSeq (Illumina) which resulted in > 2 million reads per *L. monocytogenes* field isolate. De novo genome assemblies of the reads were conducted using the SPAdes algorithm of the PATRIC database (www.patricbrc.org; Wattam et al., 2014), resulting in sequence coverage of 40- to 60-fold per consensus base for each of the six *L. monocytogenes* field isolates. Initial genome annotation was performed with the automated NCBI Prokaryotic Genome Annotation Pipeline (https://www.ncbi.nlm.nih.gov/genome/annotation_prok; Angiuoli et al., 2008).

2.4. Whole genome sequence data analysis

Whole genomic DNA sequences were analyzed using the DS Gene software package (v. 2.5; Accelrys GmbH, Munich, Germany). Based on a literature search the following genes were attributed to nisin resistance and their putative regulation sequences associated with nisin resistance were identified. Genes related to cell wall modifications: *dltA, dltB, dltC* and *dltD* (D-alanyl decoration of teichoic acid; Abi Khattar et al., 2009; Kovács et al., 2006; Peschel et al., 1999), *pbp* (lmo2229; penicillin-binding protein; Gravesen et al., 2001), and *rmlD* (lmo1084; synthesis of dTDP-L-rhamnose; Xuanyuan et al., 2010). Gene for cell membrane modifications: *mprF* (lmo1695; catalyzing lysine esterification of phosphatidylglycerol; Thedieck et al., 2006). Genes coding for two-component systems (TCA) and TCA regulators: *liaSR* (lmo1021 and lmo1022; transcriptional regulatory proteins; Collins et al., 2012; Fritsch et al., 2011), *lisRK* (involved into responses to environmental stresses; Cotter et al., 2002), and *virRS* (lmo1741 and lmo1745; regulatory proteins for resistance to cationic peptides; Mandin et al., 2005). Genes for ABC transporters or BceAB-like transporters: *anrB* (lmo2115; multidrug resistance transporter; Collins et al., 2010a), lmo1746 (ABC transporter permease) and lmo1747 (ABC transporter binding protein; Bergholz et al., 2013; Gebhard and Mascher, 2011), and *telA* (lmo1967; homologue of the tellurite resistance gene; Collins et al., 2010b; Bergholz et al., 2013). In addition, nisin susceptibility was assigned to *gadD1, gadD2, gadD3, gadT1 and gadT2* (lmo0447, lmo2363, lmo2434, lmo0448 and lmo2362; glutamate decarboxylase system; Begley et al., 2010), *arcA* (lmo0043; arginine deiminase; Kramer et al., 2006) and lmo0047 (lipoprotein; Fritsch et al., 2011). Furthermore, the genes for alternative sigma factor *sigB* and *sigL* (general stress response; Begley et al., 2006; Palmer et al., 2009) were analyzed. The nucleic acid sequences of the selected genes were retrieved from WGS data of the six *L. monocytogenes* field isolates (Table 2) and were aligned by Pustel and ClustalW to elucidate DSVs. Alignments with DS Gene (Accelrys Inc.) were carried out in case of an amino acid sequence similarity of at least 75%. Putative prophage sequences in the sequenced *L. monocytogenes* genomes were identified with the PHAST-Phage search tool (Zhou et al., 2011). Bacterial promoter and transcription factor binding sites were predicted within a 300 bp upstream region of the target genes using BPROM (Softberry Inc., NY, USA; Solovyev and Salamov, 2011). Putative Shine-Dalgarno sequences were identified by visual inspection of purine rich sequence stretches according to the consensus sequence provided by Shine and Dalgarno (1974).

2.5. Modeling of GadD2 proteins

Comparison of amino acid sequences of NR, NS and the *L. monocytogenes* EGD-e (Glaser et al., 2001; UniProt ID Q9EYW9) genomes, which was used in many other studies as reference (Bécavin et al., 2014), unveiled a C-terminal change in $D_{453}N$. Based on crystal structures of *Escherichia coli* GadD2 determined at pH 7.6 (PDB:1PMO) and pH 4.6 (PDB:1PMM), the C-terminus sterically modulates the activity of the enzyme in a pH-dependent manner (Capitani et al., 2003). In PDB:1PMM, the N-terminus forms a helix bundle, which is then associated to the membrane. The C-terminus is hinged and as a result it does not block the active site. Due to fluctuation of the C-terminus, its atom positions could not be determined for structure PDB:1PMM, but the active site was shown to be not occupied (Capitani et al., 2003). As no 3D structure data of GadD2 structure of *L. monocytogenes* was available, structure models were predicted by a combined threading and modeling approach using independently the programs EVfold (Marks et al., 2011), I-TASSER (Wang et al., 2017), M4T (Fernandez-Fuentes et al., 2007), PHYRE2 (Kelley et al., 2015), RaptorX (Källberg et al., 2012), and SWISS-MODEL (Biasini et al., 2014). Standard parameters were set for all tools. The top-scored model of each analysis was structurally aligned using PyMOL (Schrödinger, 2015), in order to see consensus in the prediction as well as to locate the DSV position spatially.

2.6. Statistics

Data of nisin susceptibility was not normal distributed within the category source of isolation and serotype. The proportions of NS and NR *L. monocytogenes* field isolates in each category were then compared by nonparametric tests. The two-sided Kruskal-Wallis test ($\alpha = 0.05$) was applied to compare two different categories (v. 3.3.3; R Studio, Vienna, Austria). For post-hoc testing, a two-sided Wilcoxon-Mann-Whitney-test with Bonferroni correction at 95% confidence interval was used. Because of the low numbers, *L. monocytogenes* field isolates of serotype IVa ($n = 4$) and without serotypic classification ($n = 5$) were omitted from statistical analysis. In addition, the non-linear correlation coefficient η (Pearson, 1911) between nisin susceptibility and serotype or isolation source was calculated. Two-sided Student's *t*-tests ($\alpha = 0.05$) were carried out of OD after 24, 48, 72, 96, 120, 144 and 168 h of incubation to test statistical significance of respective growth rates with and without nisin addition.

2.7. Nucleotide sequence accession numbers

The whole genome shotgun projects have been deposited at DDBJ/EMBL/GenBank under the accession LJBU00000000 (BfR L41), LJBV00000000 (BfR L245), LJBY00000000 (BfR L261), LJBZ00000000 (BfR L330), LJCA00000000 (BfR L448), and LJDG00000000 (BfR L1079).

3. Results

3.1. Nisin susceptibility of L. monocytogenes field isolates

The MIC_{90} value of the 282 *L. monocytogenes* field isolates was ≤ 1500 IU ml^{-1}, and seven field isolates (2.5%) were classified as NR (MIC > 1500 IU ml^{-1}; Fig. 1). Nisin susceptibility of *L. monocytogenes* field isolates was positively correlated to their source and serotype (Table 1). In addition, only *L. monocytogenes* field isolates of serotype IIa were NR (Supplementary Fig. 1). *Listeria monocytogenes* field isolates derived from milk/cheese and other dairy products were inhibited at significantly higher nisin concentrations than isolates from other sources ($p < 0.002$).

3.2. Genome sequence analyses

Four NS and two NR were selected for WGS analyses based on their respective MIC and their classification to serotype IIa (Table 2). Comparing prophage and plasmid sequences of NS and NR revealed no association with nisin susceptibility (Supplementary Tables S3 and S4). In addition, DSVs of genes necessary for cell wall modifications, BceAB-like ABC transporter, genes encoding for two-component systems (TCA) and TCA regulators, alternative sigma factors as well as regulatory sequences on the genomes showed no association with nisin susceptibility (Supplementary Tables S5 and S6). However, genomic DSV analyses

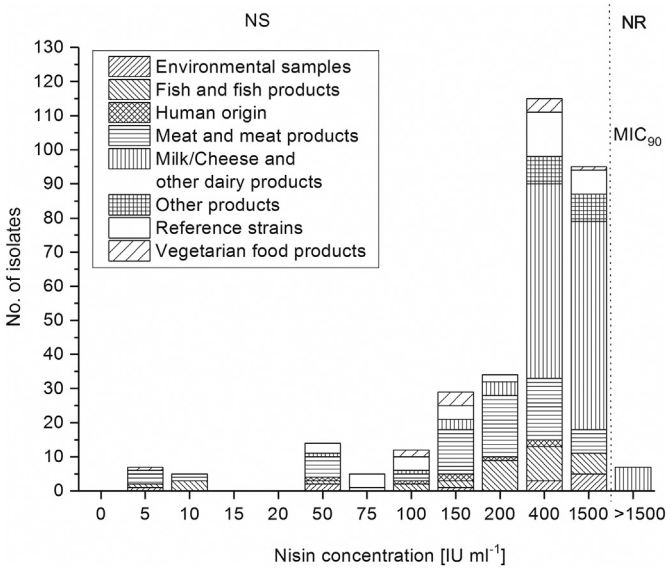

Fig. 1. Minimum inhibitory concentration (MIC) of nisin and isolation sources of 282 *L. monocytogenes* field isolates and 39 *Listeria* reference strains (see Supplementary Table S2). The MIC_{90} value was 1500 IU ml^{-1}. *L. monocytogenes* field isolates that showed higher MIC values (> 1500 IU ml^{-1}) were categorized as nisin resistant (NR) whereas nisin susceptible (NS) isolates revealed MIC values ≤1500 IU ml^{-1}.

revealed an amino acid substitution at $D_{453}N$ in *gadD2* gene present in the two NR but absent in the four NS (Table 3, Supplementary Fig. S2).

3.3. Modeling of the GadD2 protein structure

To predict structural changes of GadD2 upon $D_{453}N$ substitution the protein with amino acid substitution $D_{453}N$ was modeled based on the crystal structure of *E. coli* GadD2 as determined by Capitani et al. (2003) (Supplementary Fig. S3). The substitution $D_{453}N$ was found in a hinge region of the C-terminal tail, which bends to enter the active site in neutral pH environments (Fig. 2), rendering the active site inaccessible. The $D_{453}N$ substitution may cause a structural change that enables higher enzyme activity at both neutral and low pH conditions.

3.4. pH-dependent growth with and without sub-inhibitory nisin concentration over time

To test the effect of $D_{453}N$ substitution in GadD2, growth rate of each of the six *L. monocytogenes* field isolates were monitored over time at pH 7.0 and pH 5.5 (data not shown). Overall, both NR strains achieved in the presence of nisin significantly faster the exponential growth phase compared to all four NS strains after 24 h at pH 7.0 ($p < 0.05$). Subsequently incubation time points did not significantly differ between NR and NS strains ($p < 0.05$). At pH 5.5, however, no significant difference was observed between NR and NS in the initial and subsequent incubation time points ($p < 0.05$).

4. Discussion

The majority of the *L. monocytogenes* field isolates were characterized as NS, which is in line with previous studies that reported NS between 1.85 and 2000 IU ml^{-1} (Benkerroum and Sandine, 1988; Ferreira and Lund, 1996; Iancu et al., 2012; Katla et al., 2003; Mota-Meira et al., 2000; Rasch and Knøchel, 1998; Ukuku and Shelef, 1997). However, a general definition of nisin susceptibility with cut off values as present for many antimicrobials have so far not been defined by European Committee on antimicrobial susceptibility testing (EUCAST) or others. The source of isolation was associated with NR ($\eta = 0.764$), and the majority of NR were retrieved from milk/cheese and other dairy products (Fig. 1).

Previous studies demonstrated that resistance to bacteriocins can be induced in defined food environments, selecting for sub-populations with higher nisin tolerance (Gravesen et al., 2002; Harris et al., 1991; Ming and Daeschel, 1993; Wu et al., 2017). In laboratory experiments the NR state of *L. monocytogenes* strain was acquired spontaneously and occurred at a frequency of 10^{-6}–10^{-8} (Harris et al., 1991; Ming and Daeschel, 1993).

NR was only found in serotype IIa (Supplementary Fig. 1), which is in accordance with previous studies (Buncic et al., 2001; Katla et al., 2003). In contrast, a comparable surveillance study on *L. monocytogenes* field isolates from pork and beef revealed no association of NR and serotype (Rasch and Knøchel, 1998). WGS is an appropriate approach to link nisin susceptibility to selected genotypes of the same serotype as described already in other settings (Hingston et al., 2017; Jahn et al., 2017). The majority of coding genes and regulatory elements known to be related to nisin resistance as well as prophages were not associated to NR in our study except for one DSV in the *gadD2* gene (Table 3,

Table 3
Comparison of the *gad* operon in nisin susceptible (NS) and nisin resistant (NR) *L. monocytogenes* field isolates (referred to BfR L41). The amino acid substitution in GadD2 (453: D → N) is marked in bold.

Protein (size)	Predicted function	NS *L. monocytogenes* field isolates				NR *L. monocytogenes* field isolates	
		BfR L41	BfR L330	BfR L448	BfR L1079	BfR L245	BfR L261
GadD1 (463 aa)	Catalyze decarboxylation of glutamate	+	n.d.	n.d.	+	n.d.	n.d.
GadD2 (464 aa)	Catalyze decarboxylation of glutamate	–	380: N → D	380: N → D	–	–	–
		–	385: K → N	385: K → N	–	–	–
		–	–	–	–	**453: D → N**	**453: D → N**
		–	454: T → N	–	–	–	–
GadD3 (467 aa)	Catalyze decarboxylation of glutamate	–	–	–	–	–	145: E → K
		–	187: N → D	–	–	–	187: N → D
		–	207: V → I	–	–	–	207: V → I
		–	–	353: L → I	–	353: L → I	–
GadT1 (484 aa)	Antiporter in decarboxylation of glutamate	+	n.d.	n.d.	+	n.d.	n.d.
GadT2 (507 aa)	Antiporter in decarboxylation of glutamate	–	–	–	278: G → D	–	–
		–	–	409: V → I	–	409: V → I	–
		–	–	–	–	416: V → I	–
		–	–	419: V → I	419: V → I	–	–
		–	–	438: M → T	438: M → T	–	–
		–	–	441: I → M	441: I → M	–	–

Abbreviations: NS, nisin susceptible; NR, nisin resistant.

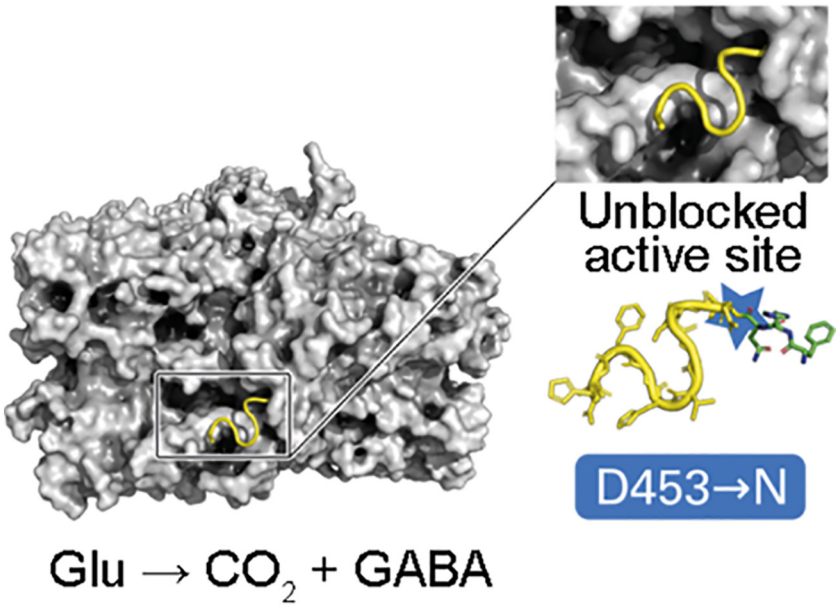

Fig. 2. The modeled protein structure of GadD2 with substitution $D_{453}N$ of nisin resistant L. *monocytogenes* field isolates in the hinge region of the C-terminus. Modeled GadD2 protein structure with substitution $D_{453}N$ decarboxylates glutamate (Glu) to γ-aminobutyric acid (GABA) and is less pH dependent.

Supplementary Tables S3–6). By comparing *gadD2* sequences of NS and NR, a substitution $D_{453}N$ in the hinge region of the C-terminus restricted to NR was identified (Fig. 2). The modeled protein structure of GadD2 with substitution $D_{453}N$ indicates that the C-terminus could not block the active site at pH 7.0 (Supplementary Fig. 3). In contrast, active site was blocked in the modeled protein structure without substitution as found in NS. Moreover, this substitution $D_{453}N$ caused a significant prolonged lag phase for NS but not for NR in the presence of nisin at pH 7.0. However, GAD assays based on a colorimetric assay of pH shift with standardized inoculum in the presence and absence of nisin for NS and NR and *Lactobacillus fermentum*, as GAD negative control, and *E. coli*, as GAD positive control, in three different culture broths did show inconsistent results as was also reported by Djoko et al. (2017) (data not shown).

The relevance of the GAD system of *L. monocytogenes* to resist elevated nisin concentrations has been described frequently (Bearson et al., 2009; Begley et al., 2010; Caballero Gómez et al., 2013). Begley et al. (2010) showed that an isogenic Δ*gadD1* mutant of *L. monocytogenes* LO28 resulted in NS state compared to the parent NR state. In the same study, NR benefited from additional ATP formation via the γ-aminobutyric acid shunt pathway, in which GadD2 is involved (Begley et al., 2010). In agreement with Capitani et al. (2003), who studied the activation of GadD2 in *E. coli*, our study describes a reduced pH-dependence of GadD2 in *L. monocytogenes* by substitution $D_{453}N$, which may lead to an increased tolerance to acidic stress. Hence, the $D_{453}N$ substitution is probably responsible for decreased nisin susceptibility by supplying ATP (Begley et al., 2010; Collins et al., 2011) to counteract the nisin-induced pore formation of the cell membrane (Ruhr and Sahl, 1985).

Sequence coverage in WGS of *gadD1* was to low and we therefore excluded *gadD1* from further analyses. In addition, most serotype IVb strains lack the *gadD1* gene (Chen et al., 2012; Cotter et al., 2005) indicating a lower likelihood of NR in this serotype. None of the amino acid substitutions observed in the *gadD3* gene could be attributed to NR or nisin susceptibility.

Both NRs for WGS analyses were isolated from raw milk (Table 2), which pH is usually maintained neutral. As raw milk is very often accompanied by nisin-producing lactic acid bacteria, *L. monocytogenes* with substitution $D_{453}N$ appeared to maximize protection at neutral pH with their GAD system in combination with other nisin resistance mechanisms. In absence of this substitution the proton motive force may deplete faster due to blocked GadD2 as predicted by its modeled protein structure.

Many genotypes and gene regulations have been attributed to NR and were not confirmed in this study. The substitution $D_{453}N$ in the GAD system was supportive to reduce the lag phase in the presence of nisin at pH 7.0. However, the NR is manifold and upcoming proteome or transcriptome analyses or knock-mutant studies will hopefully improve our understanding of NR in *L. monocytogenes*.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

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