Sublethal Triclosan Exposure Decreases Susceptibility to Gentamicin and Other Aminoglycosides in *Listeria monocytogenes*[▽]

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The human food-borne pathogen *Listeria monocytogenes* is capable of persisting in food processing plants despite cleaning and sanitation and is likely exposed to sublethal biocide concentrations. This could potentially affect susceptibility of the bacterium to biocides and other antimicrobial agents. The purpose of the present study was to determine if sublethal biocide concentrations affected antibiotic susceptibility in *L. monocytogenes*. Exposure of *L. monocytogenes* strains EGD and N53-1 to sublethal concentrations of Incimaxx DES (containing peroxy acids and hydrogen peroxide) and Triquart Super (containing quaternary ammonium compound) in four consecutive cultures did not alter the frequency of antibiotic-tolerant isolates, as determined by plating on 2× the MIC for a range of antibiotics. Exposure of eight strains of *L. monocytogenes* to 1 and 4 µg/ml triclosan did not alter triclosan sensitivity. However, all eight strains became resistant to gentamicin (up to 16-fold increase in MIC) after exposure to sublethal triclosan concentrations. Gentamicin-resistant isolates of strains N53-1 and 4446 were also resistant to other aminoglycosides, such as kanamycin, streptomycin, and tobramycin. Gentamicin resistance remained at a high level also after five subcultures without triclosan or gentamicin. Aminoglycoside resistance can be caused by mutations in the target site, the 16S rRNA gene. However, such mutations were not detected in the N53-1-resistant isolates. A combination of gentamicin and ampicillin is commonly used in listeriosis treatment. The triclosan-induced resistance is, hence, of great concern. Further investigations are needed to determine the molecular mechanisms underlying the effect of triclosan.

Listeria monocytogenes is a food-borne human pathogen that can cause the highly fatal infection listeriosis. The number of listeriosis cases has increased in recent years in several European countries, including Denmark (2, 14). The reason(s) for this increase is not known, but it has been suggested that changes in antibiotic therapy of patients with sepsis, increase in exposure to *L. monocytogenes* (e.g., due to increase in consumption of ready-to-eat foods), or alterations of strain virulence could be the cause (14). Listeriosis is commonly treated with the antibiotics ampicillin or penicillin G in combination with an aminoglycoside—usually gentamicin (34). If the disease is diagnosed in time, this treatment is usually effective; however, as for other bacteria, development of antibiotic resistance is of great concern. Generally, antibiotic resistance in *L. monocytogenes* is uncommon. However, the frequency of antibiotic-resistant isolates in foods has been increasing (22). This is alarming as listeriosis predominantly occurs following consumption of contaminated foods (10).

In the food processing industry and clinical settings, disinfection with biocides is used to reduce or eliminate microorganisms. The construction of the food processing equipment is often complex, and not all organic material may be removed during the cleaning process. Hence, the efficiency of the subsequent biocide treatment will be hampered, and the bacterial cells may be exposed to sublethal biocide concentrations only. This can affect the bacterial cell, and we have recently dem-

onstrated that low nonlethal biocide concentrations influence virulence gene expression in *L. monocytogenes* (18). Furthermore, it has been hypothesized that such sublethal exposure may potentially affect both biocide and antibiotic susceptibility.

Previous studies have examined possible links between biocide exposure or biocide resistance and changed antibiotic susceptibility. Aase et al. (1) found that *L. monocytogenes* strains that were tolerant to the biocide benzalkonium chloride (BC) (2× the MIC) did not have changed antibiotic susceptibility compared to BC-sensitive strains. However, Romanova et al. (32) found that BC-adapted *L. monocytogenes* had a 2- to 4-fold increase in gentamicin and kanamycin MICs compared to the wild-type strains. It was suggested that the increased MIC of BC in the adapted strains was caused by increased expression of the efflux pump-encoding gene *mdrL*, and this could also be the cause of the changes in antibiotic susceptibility (32). In addition, biocide adaption or exposure caused decreased antibiotic susceptibility in *Salmonella enterica* serovar Virchav, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (5, 7, 36). Given the indications that biocide exposure can alter antibiotic susceptibility, there is a clear need for further investigation, especially of a bacterium such as *L. monocytogenes* that is likely exposed to biocides both in the clinical setting and in the food processing industry.

Specific molecular subtypes of *L. monocytogenes* are able to persist within different types of food processing plants, and they may repeatedly (over years) be isolated from the same environment (21, 30, 41, 42). The mechanisms that enable persistence are not known; however, the residing bacteria are likely food product contaminants and are also repeatedly exposed to biocides. It is therefore particularly important to determine if persistent strains are affected by biocide exposure.

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TABLE 1. Origin, RAPD type, serotype, and lineage of the *Listeria monocytogenes* strains used in the present study

Strain	Origin	Frequently occurring persistent RAPD ^a type in processing plants	RAPD type	Serotype	Lineage	Source or reference
N53-1	Smokehouse equipment	Yes	9	1/2a	2	42
La111	Cold-smoked salmon	Yes	9	1/2a	2	40
V518a	Smokehouse equipment	Yes	15	4b	1	41
7291	Pasta with chicken	Yes	15	4b	1	20
N22-2	Floor in smokehouse	No	48	1/2a	2	39, 42
EGD	Rabbit ^b	No	68	1/2a	2	W. Goebel
4446	Human, clinical	No	71	1/2b	1	20
Br22	Sediment in freshwater fish tank	No	X ^c	1/2a	2	13

^a RAPD, randomly amplified polymorphic DNA.

^b This strain was originally discovered in 1926 and was kindly provided by Werner Goebel, University of Würzburg.

^c The RAPD type is different from the persistent RAPD types from processing plants.

In the present study, we exposed eight strains of *L. monocytogenes* to sublethal concentrations of biocides and determined if their subsequent antibiotic susceptibility was altered. We chose two industrial disinfectants, Incimaxx DES (a peroxy acid- and hydrogen peroxide-containing biocide) and Triquart Super (a quaternary ammonium compound [QAC]-containing biocide), containing active ingredients that are routinely used in the food industry. The peroxygens acts as oxidants by producing radicals that attack essential cell components, including lipids, proteins, and DNA, and they decompose to safe by-products (26). QAC is a cationic, membrane-active component that targets the cytoplasmic membrane of bacteria, causing loss of structural organization and integrity of the cytoplasmic membrane (26). Also, we included triclosan [5-chloro-2-(2,4-dichlorophenoxy)phenol], which is a widely used broad-spectrum biocide. Triclosan has, unlike other biocides, a specific target when used at sublethal concentrations, namely, an enoyl-acyl carrier protein (ACP) reductase isoform, FabI (28). Additional types of triclosan-mediated bactericidal activity, such as interruption of membrane integrity and interference with respiration, have been suggested (4, 38). The mechanism of action of lethal triclosan concentrations has, to our knowledge, not been described. Triclosan is incorporated into many different products, from soaps, to towels, to baby toys and even food storage containers (43). In Europe, triclosan must not be used as a disinfectant in food and feed production, but incorporation into cosmetics, including hand soaps and toothpaste, is not prohibited (35). The wide distribution and stability of triclosan have resulted in increased environmental exposure (33). As *L. monocytogenes* is a ubiquitous environmental bacterium, it may likely be exposed to triclosan in its different habitats.

In the present study, we determined how sublethal biocide exposure affected antibiotic resistance in eight strains of *L. monocytogenes* of environmental, food, and clinical origins. The study included strains isolated as persisting subtypes in the food industry since we hypothesized that the persistent subtypes regularly had been exposed to sublethal concentrations of biocides when residing in the food processing industry and potentially could respond differently to biocide exposure.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. Eight *L. monocytogenes* strains (Table 1), representing different serotypes, lineages, and origins, were used in the present study. Strains N53-1, La111, and V518a were isolated from fish processing industries and represent molecular subtypes that frequently occur as persistent subtypes in these industries (40–42). Strain N22-2 was isolated from a fish smokehouse (42), and Br22 was isolated from sediment in a freshwater fish tank (13). Both strains represent molecular subtypes occurring only sporadically in the food industry. A human clinical isolate, 4446 (20), and an animal isolate, EGD, were also included. Stock cultures were stored in 4% (wt/vol) glycerol, 3% (wt/vol) tryptone soya broth (BD catalog no. 211705), 2% (wt/vol) skim milk powder (BD catalog no. 232100), and 0.5% (wt/vol) glucose at -80°C . *L. monocytogenes* cells were cultured on brain heart infusion (BHI) agar (Oxoid; CM1135 supplemented with 1.5% agar [VWR catalog no. 20768.292]) at 30°C for 2 to 3 days or at 37°C overnight. A single colony was inoculated into BHI broth at 37°C and grown overnight with shaking (250 rpm), followed by $1,000\times$ dilution and incubation for an additional 18 h, reaching a density of 10^9 CFU/ml. Antibiotic-tolerant *L. monocytogenes* strains were selected and cultured on BHI agar supplemented with antibiotics in concentrations of $2\times$ the wild-type MIC at 37°C for 2 days.

Preparation of antibacterial agent solutions. Triclosan (Irgasan; Sigma-Aldrich catalog no. 72779) was dissolved in 70% ethanol when used for growth experiments and in 99% ethanol when used for MIC determinations. The peroxy acid- and hydrogen peroxide-containing biocide Incimaxx DES (Ecolab, Denmark) and the quaternary ammonium compound-containing biocide Triquart Super (Ecolab, Denmark) were diluted in sterilized, demineralized water. Biocide solutions were stored at 5°C for up to 7 days. The antibiotics ampicillin (Sigma-Aldrich catalog no. A9518), gentamicin (Sigma-Aldrich catalog no. G3632), kanamycin (Sigma-Aldrich catalog no. K4000), penicillin G (Sigma-Aldrich catalog no. 13752), spectinomycin (Sigma-Aldrich catalog no. 85555), streptomycin (Sigma-Aldrich catalog no. S6501), tetracycline (Sigma-Aldrich catalog no. T3383), and tobramycin (Sigma-Aldrich catalog no. T1783) were dissolved in sterilized, demineralized water. Rifampin (Sigma-Aldrich catalog no. R3501) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich catalog no. D5879). Ampicillin stock was stored at -70°C , and rifampin and tobramycin stocks were stored at -20°C . The remaining antibiotic stocks were stored at 5°C .

Antibiotic MICs on solid media. BHI agar was supplemented with 0.125 to 32 $\mu\text{g/ml}$ ampicillin, 2 to 128 $\mu\text{g/ml}$ gentamicin, 0.019 to 20 $\mu\text{g/ml}$ rifampin, or 2.5 to 160 $\mu\text{g/ml}$ streptomycin. Plates with no antibiotics were supplemented with sterilized, demineralized water (the solvent used for antibiotics). One hundred microliters of outgrown bacterial culture (10^9 CFU/ml) was plated on antibiotic-containing BHI agar and incubated at 37°C for 48 h. The MICs of ampicillin and streptomycin were set at the lowest antibiotic concentration with no growth on the agar plates. Even at high gentamicin and rifampin concentrations, a few colonies developed on the agar plates, and the MIC was set at the antibiotic concentrations differentiating between plates with very few colonies and overgrown plates. The MICs for all four antibiotics were determined in successive experiments, increasing the increment between concentration steps when necessary.

Determination of sublethal biocide concentrations. Outgrown cultures of *L. monocytogenes* EGD and N53-1 were diluted to 10^4 CFU/ml and exposed to a

dilution series of biocides. Biocides were added to final concentrations of 0.0075 to 0.5% (vol/vol) Incimaxx DES, 0.25 to 64 µg/ml triclosan, or 0.0001 to 0.0022% (vol/vol) Triquart Super. Cultures with no biocide were exposed to the relevant solvent. Cultures were incubated at 37°C with shaking (250 rpm) for 24 h, and the number of CFU was determined on BHI agar. Concentrations that caused no or marginal reduction and a 1- or 2-log reduction in cell density, respectively, were used in subsequent experiments. Reproducibility was evaluated by conducting the above procedure independently up to four times for each strain and biocide concentration.

Exposure to sublethal biocide concentrations and testing for change in antibiotic susceptibility. *L. monocytogenes* EGD and N53-1 were grown in the following sublethal biocide concentrations: 0.015% (vol/vol) and 0.125% (vol/vol) Incimaxx DES for EGD, 0.062% (vol/vol) and 0.25% (vol/vol) Incimaxx DES for N53-1, 0.0004% (vol/vol) and 0.0008% (vol/vol) Triquart Super for EGD, and 0.0011% (vol/vol) and 0.0022% (vol/vol) Triquart Super for N53-1. Subsequently, all eight *L. monocytogenes* strains were exposed to 1 and 4 µg/ml triclosan.

All cultures were diluted to 10⁴ CFU/ml and grown with or without sublethal biocide concentrations for 24 h at 37°C with shaking (250 rpm). After 24 h, the cultures were diluted to 10⁴ CFU/ml and regrown in sublethal biocide concentrations. In total, four consecutive 24-h cultures in sublethal concentrations of biocides were done for each strain. Cell density was determined at the start of each subculture and on the overnight culture by plating on BHI agar. To determine if the biocide exposure altered antibiotic resistance, we also plated all outgrown cultures on BHI agar with 2× the MIC of ampicillin, gentamicin, rifampin, or streptomycin.

Antibiotic resistance frequency was calculated by dividing the number of CFU/ml on antibiotic plates by the number of CFU/ml on BHI agar. The bacterial cell concentration of antibiotic-resistant isolates was set at 1 CFU/ml if below the detection limit of 10 CFU/ml.

A maximum of eight tolerant isolates per strain per concentration were restreaked and cultured on antibiotic-containing BHI agar. Isolates of all eight *L. monocytogenes* strains that were used in subsequent experiments were verified as *Listeria* by cultivation on *Listeria* selective agar base (Oxoid catalog no. CM0856) supplemented with modified *Listeria* selective supplement (Oxoid catalog no. SR0206E) at 30°C for 48 h. Subsequently, the 16S rRNA gene of several N53-1 isolates was sequenced (see below), which also served as the species verification. Isolates were cultured at 37°C in BHI broth supplemented with antibiotics at 1/2× the MIC or the MIC (wild type) and stored in 15% glycerol at -80°C.

Strains EGD and N53-1 were cultured in one experiment with Incimaxx DES and Triquart Super and in two experiments with triclosan (verifying the appearance of gentamicin-tolerant colonies). Subsequently, the six remaining *L. monocytogenes* strains were exposed to triclosan and tested for gentamicin tolerance.

Antibiotic and triclosan MICs in liquid media. Antibiotic and triclosan susceptibilities in liquid media were determined for both wild-type and antibiotic-tolerant *L. monocytogenes* isolates by a broth microdilution method (8). Two-fold dilution series were made of the antibacterial compounds in BHI broth in microtiter plates (Nunc A/S catalog no. 163320). The concentrations were 0.08 to 640 µg/ml gentamicin, 0.25 to 512 µg/ml kanamycin, 0.03 to 64 µg/ml penicillin G, 0.5 to 4,096 µg/ml spectinomycin, 0.17 to 1,280 µg/ml streptomycin, 0.03 to 64 µg/ml tetracycline, 0.06 to 128 µg/ml triclosan, and 0.03 to 64 µg/ml tobramycin. Controls were either BHI alone or BHI with ethanol (the solvent of triclosan). The colony mass of *L. monocytogenes* grown on BHI agar with or without gentamicin (2× the MIC of the wild type) was dissolved in BHI broth to an optical density at 546 nm (OD₅₄₆) of 0.13 (approximately 10⁸ CFU/ml) (11) (Novaspec II visible spectrophotometer; Pharmacia Biotech), and the MIC was determined using a starting concentration of 5 × 10⁵ CFU/ml in the microtiter plates. Microtiter plates were incubated for 20 to 22 h at 37°C. Cultures from 1/2× the MIC were streaked on BHI agar and cultured at 37°C overnight to check purity.

The MIC of triclosan was determined in duplicate for all eight wild-type strains. The MIC of gentamicin was determined in duplicate or triplicate in at least two independent experiments for all eight wild-type strains. The MICs of gentamicin and triclosan were determined in duplicate for gentamicin-tolerant isolates. The MICs of kanamycin, penicillin G, spectinomycin, streptomycin, tetracycline, and tobramycin were determined in double determinations for N53-1 and 4446 and once for the selected gentamicin-tolerant N53-1 and 4446 isolates.

Determination of fitness cost of antibiotic resistance. Potential fitness cost and ability to grow under gentamicin stress were determined for *L. monocytogenes* N53-1 gentamicin-tolerant isolates. An outgrown culture of N53-1 was prepared in BHI broth, whereas N53-1-tolerant isolates were grown in BHI broth with gentamicin (10 µg/ml). Approximately 5 × 10⁵ CFU/ml was inoculated in BHI

with or without 20 µg/ml gentamicin (2× the MIC for N53-1). The OD₆₀₀ was determined every hour until the stationary phase.

Sequencing of aminoglycoside binding site. Gentamicin resistance can be caused by a single point mutation in the 16S rRNA gene, and we therefore sequenced this gene from a number of the tolerant N53-1 isolates to determine if this was the cause of the triclosan-induced gentamicin resistance. Overnight cultures of gentamicin-resistant N53-1 isolates were prepared in media supplemented with 20 µg/ml gentamicin. Chromosomal DNA was purified from overnight cultures using the Dynabeads DNA direct universal kit (Invitrogen catalog no. 630.06) following the manufacturer's recommendations. *L. monocytogenes* carries four to six alleles of the 16S rRNA subunit determined by NCBI genome database search (<http://www.ncbi.nlm.nih.gov/sites/genome>). Using the genome sequence for EGD-e (<http://www.ncbi.nlm.nih.gov/sites/genome>; GenBank accession no. A1591824) as a template, approximately 1,150 to 1,550 bp of 16S rRNA alleles and 300 bp downstream of the genes were amplified from EGD, N53-1, and gentamicin-resistant N53-1 isolates. The primers 16S rRNA FW1 (CAGCTCGTGTCTGTGAGATGT), 16S rRNA BW1-1 (CGTGCGCCCTTCTAAGT), and 16S rRNA BW2 (TCGACCTCAGCTTATCAGG) were used. PCR with 16S rRNA FW1 and 16S rRNA BW1-1 amplified the first four EGD-e alleles (Imor01, Imor04, Imor09, and Imor12), while 16S rRNA FW1 and 16S rRNA BW2 were used to amplify alleles 5 and 6 (Imor15 and Imor18). PCR was conducted with 1× Hot Start PCR buffer (Fermentas Life Science catalog no. EP0602), 0.4 µM forward primer, 0.4 µM reverse primer, 4 mM MgCl₂ (Fermentas Life Science catalog no. EP0602), 0.2 mM deoxynucleoside triphosphate (dNTP) (Roche catalog no. 11969064001), 0.025 U/µl Maxima hot start polymerase (Fermentas Life Science catalog no. EP0602), and 2 µl chromosomal DNA in a total volume of 25 µl. The amplification was conducted with initial denaturation at 95°C for 4 min, 40 cycles with denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were evaluated on 1% agarose gel. The Quick Load 100-bp DNA ladder (Biolabs catalog no. NO467S) was used as a standard. PCR products were eluted from the gel using the GFX PCR DNA and gel band purification kit (GE Healthcare catalog no. 28-9034-70). Sequencing was conducted at Eurofins MWG Operon, Germany, using the primers 16S rRNA FW1, 16S rRNA BW1-1, and 16S rRNA BW2. Sequenced segments were assembled in DNA base (Heracle Biosoft) and aligned in ClustalX (UCD Dublin). Alignment illustrations were edited in BioEdit (Ibis Therapeutics).

Statistical analysis. Antibiotic resistance frequency was log₁₀ transformed. A paired *t* test with a significance level of *P* < 0.05 was used to determine if the gentamicin resistance frequency of all eight strains combined was influenced by exposure to triclosan after 4 days (4 × 24 h). Statistical analysis was done with GraphPad Prism (GraphPad Software, Inc.).

RESULTS

Exposure to sublethal biocide concentrations and testing for change in antibiotic susceptibility. Two *Listeria monocytogenes* strains (EGD and N53-1) were grown in two sublethal concentrations of Incimaxx DES (a peroxy acid- and hydrogen peroxide-containing biocide) and Triquart Super (a quaternary ammonium compound-containing biocide). Four successive transfers at sublethal concentrations did not change the number of antibiotic-resistant colonies of the strains on BHI agar with ampicillin, gentamicin, or streptomycin (data not shown). The rifampin resistance frequencies were similar in the controls and the biocide-exposed cultures. Spontaneous mutations were considered the cause and were not investigated any further. However, exposure to sublethal concentrations of triclosan resulted in an increase in the frequency of resistant colonies on gentamicin plates, and we pursued this observation in more detail.

Triclosan exposure and changes in antibiotic susceptibility. Triclosan at 1 and 4 µg/ml caused no or only marginal reduction in maximal cell counts during transfer on 4 consecutive days (Fig. 1). Plating of the triclosan-exposed *L. monocytogenes* EGD and N53-1 cultures caused only a minor change in the number of colonies on BHI agar supplemented with ampicillin

FIG. 1. Bacterial counts of *L. monocytogenes* N53-1 cultured with 4 µg/ml (◆), 1 µg/ml (□), or 0 µg/ml (▲) triclosan for 4 × 24 h with reinoculation every 24 h. The results presented are representative of two independent experiments.

or streptomycin at concentrations of 2× the MIC for the wild type (data not shown). Triclosan-exposed cultures had a number of colonies on rifampin-containing media similar to that of the control cultures (data not shown). However, when EGD and N53-1 were exposed to sublethal concentrations of triclosan, the frequency of colonies on gentamicin plates increased compared to that of the controls by 10- to 1,000-fold following 4 × 24 h of triclosan exposure (Table 2). Several *L. monocytogenes* strains were subsequently exposed to triclosan and plated on BHI agar containing gentamicin (2× the MIC of the wild type). The increase in gentamicin resistance frequency following triclosan exposure was observed for all *L. monocytogenes* strains (Table 2). The gentamicin resistance frequency increased 10- to 10,000-fold after exposure to 1 and 4 µg/ml and was significantly higher than that of the controls ($P = 0.003$ at 1 µg/ml and $P = 0.004$ at 4 µg/ml). The increase in resistance frequency appeared to be independent of the triclosan concentration (i.e., 1 or 4 µg/ml; $P = 0.55$) (Table 2) and exposure time (data not shown).

The gentamicin resistance frequency also increased in some control cultures of strains N53-1, La111, N22-2, EGD, and 4446 upon triclosan exposure (data not shown) (Table 2); however, it was always at a much lower level than in the triclosan-exposed cultures.

MICs of gentamicin and triclosan and colony morphology of selected isolates. We selected up to eight gentamicin-tolerant isolates per strain per triclosan concentration, and those used for further studies were all verified as *Listeria* on Oxford agar.

The MICs of gentamicin and triclosan were determined for the isolates selected after 4 × 24 h of triclosan exposure. The impact of selective pressure was evaluated by determining the MICs for isolates pregrown with or without gentamicin. The triclosan MIC was unaffected by triclosan exposure or the presence or absence of gentamicin in preculture (Table 3). Isolates pregrown with gentamicin had a 4- to 16-fold increase in gentamicin MIC compared to the wild type (Table 3). The gentamicin MIC remained at the high level also when isolates were pregrown without gentamicin (Table 3). Removal of gentamicin from the preculture caused a decrease in gentamicin MIC for one N53-1 isolate that was selected from the control (non-triclosan-exposed) cultures (Table 3).

The gentamicin MIC was determined for isolates of N53-1 following five consecutive subcultivations on BHI agar without gentamicin to determine if the resistance would disappear without the selective pressure. Gentamicin resistance was

maintained in all triclosan-exposed isolates for all five subcultivations except one (Table 4). The isolate selected from the control was as susceptible to gentamicin as the wild type after a single subcultivation without gentamicin (Tables 3 and 4).

The N53-1 isolates selected from 4 µg/ml triclosan (2-2) and the control culture (1-1) had the same colony size as the wild type on BHI agar, whereas the two isolates selected from 1 µg/ml triclosan (1-1 and 2-1) were small colonies. This morphology was observed on BHI agar with and without gentamicin (16 µg/ml). No difference on cell morphology was observed when studied by light phase-contrast microscopy (×1,000 magnification).

MIC of antibiotics for gentamicin-resistant isolates. Susceptibility to other antibiotics with the same main target as gentamicin (kanamycin, spectinomycin, streptomycin, tetracycline, and tobramycin) or a distinctly different target (penicillin G) was determined for *L. monocytogenes* N53-1 and 4446 and the gentamicin-resistant N53-1 and 4446 isolates.

The MICs of penicillin G, spectinomycin, and tetracycline were similar for all strains for wild-type compared to gentamicin-resistant isolates (Table 4). However, one of the 4446 gentamicin-resistant isolates had a 4-fold decrease in MIC of tetracycline compared to the wild type. In contrast, the MICs were increased for kanamycin and tobramycin in the N53-1 and 4446 gentamicin-resistant isolates selected after triclosan exposure and also for streptomycin in the N53-1 gentamicin-resistant isolates (Table 4). The fold increase in MIC was similar to the gentamicin MIC increase (Table 4). The MICs of kanamycin and tobramycin were as stable as the MIC of gentamicin in the isolates, as similar changes in MIC occurred during subcultivation without gentamicin (data not shown). The N53-1 isolate selected from the control was as susceptible

TABLE 2. Gentamicin resistance frequency of *L. monocytogenes* after 4 × 24 h of triclosan exposure

Strain	Expt no.	Resistance frequency (log ₁₀ CFU/ml) at triclosan concn of ^a :		
		0 µg/ml	1 µg/ml	4 µg/ml
N53-1	1	-7.85	-5.01	-7.62
	2	-9.56 ^b	-8.10	-5.97
La111		-7.27	-5.98	-5.67
V518a		-9.32 ^b	-8.92 ^b	-7.68
7291		-9.26 ^b	-9.01 ^b	-5.63
N22-2		-7.56	-5.24	-8.36
EGD	1	-9.26 ^b	-9.24 ^b	-8.25 ^b
	2	-7.54	-5.56	-5.63
4446		-9.31 ^b	-4.98	-5.13
Br22		-9.45 ^b	-6.99	-5.11

^a Resistance frequency was calculated by dividing the number of CFU/ml on antibiotic plates by the number of CFU/ml on BHI agar. Resistance frequency is significantly higher due to exposure to 1 and 4 µg/ml triclosan than that of the control.

^b Cell counts of gentamicin-resistant isolates were below the detection limit of 10 CFU/ml and set at 1 CFU/ml.

TABLE 3. MICs of gentamicin and triclosan for *L. monocytogenes* pregrown without or with gentamicin^a

Strain	Triclosan exposure concn (μg/ml)	Isolate no.	MIC (μg/ml) for isolates pregrown ^a :			
			-GEN		+GEN	
			GEN	TRIC	GEN	TRIC
N53-1	0 (wt) ^d		5–10 ^b	8–16 ^b	NG	NG
	4	2-2	40–80 ^b	8	80	8
	1	1-1	80	8	80	8
		2-1	80	8	80	8
	0 (control)	1-1	10 ^c	8	80	8
La111	0 (wt)		10 ^c	8	NG	NG
	4	1	40	4–8 ^b	80	4
	1	1	40	4	40	4
	0 (control)	1	40	4	80	4
		2	80	4	80	4
		3	40	4	80	4
		4	20	4	40	4
V518a	0 (wt)		20	8	NG	NG
	4	1	160	4	160	4–8 ^b
7291	0 (wt)		10 ^c	8–16 ^b	NG	NG
	4	1	80	4	160	4–8 ^b
N22-2	0 (wt)		20 ^c	8–16 ^b	NG	NG
	4	1	10	8	80	4–8 ^b
	1	1	40	4	40	4
	0 (control)	1	80	4	80	8
		2	40	4	40	4
EGD		3	80	8	80	8–16 ^b
	0 (wt)		5 ^c	8–16 ^b	NG	NG
	4	2-1	80	16	80	8
	1	2-1	80	8	80	4
	0 (control)	2-1	40–80 ^b	16	80	8
		2-2	80	8	40	8
		2-3	80	8	40	4
4446		2-4	80	8	40	4
	0 (wt)		20 ^c	8	NG	NG
	4	1	40	8	80	8
Br22	1	1	80	8	80	8
	0 (wt)		10–20 ^b	8	NG	NG
	4	1	40	4	20–40 ^b	4
	1	1	40	8	40	8

^a GEN, gentamicin; TRIC, triclosan. One isolate per triclosan concentration was tested. Up to five isolates were tested for each control. The MIC was determined after 20 to 22 h at 37°C. NG, no growth. All isolates were tested in duplicates.

^b Ranges representing MICs differing by 2-fold when determined.

^c Medians of determined MICs are shown.

^d wt, wild type.

to kanamycin, streptomycin, and tobramycin as the wild type after a single subcultivation without gentamicin (Table 4).

Determination of fitness cost of antibiotic resistance. All of the resistant N53-1 isolates had an increased generation time during culture in BHI broth compared to the wild type (Table 5). Notably, the isolate selected from the control had a much higher generation time than the other isolates and the wild type. The presence of gentamicin stress further increased the generation time (Table 5). No growth was (obviously) observed in the wild type when grown with gentamicin (2× the MIC).

Sequencing of the 16S rRNA-encoding region. Gentamicin, kanamycin, streptomycin, and tobramycin are all aminoglycosides. A point mutation within the binding site of the aminoglycoside binding site, the 16S rRNA ribosomal subunit, could be the cause of the resistance. The aminoglycoside binding site in the 16S rRNA gene (bp 1400 to 1500) was therefore amplified, sequenced, and aligned for EGD, N53-1, and the resistant N53-1 isolates. *L. monocytogenes* carries four to six alleles of the 16S rRNA gene. Using EGD-e as a template, two primer sets amplifying alleles 1 to 4 (Imor01, Imor04, Imor09, and Imor12) and alleles 5 and 6 (Imor15 and Imor18), respectively, were constructed. Differences between alleles that advanced in the region downstream of the genes made differentiation possible. No single mutations were identified within the 16S rRNA binding site in the N53-1 gentamicin-resistant isolates for either allele (data not shown), excluding single point mutations in the aminoglycoside binding site as the cause of the resistance.

DISCUSSION

The food-borne human pathogen *Listeria monocytogenes* developed resistance to gentamicin and other aminoglycosides following exposure to the widely used biocide triclosan. Other commonly used biocide agents, such as a mixture of peroxy acid and hydrogen peroxide or quaternary ammonium compounds (QACs), did not cause a similar change in antibiotic resistance. Differences in the composition or the number of cellular targets of the biocides could have caused the different antibiotic resistance frequencies determined in the present study. Incimaxx DES and Triquart Super contain a mixture of chemical compounds having multiple targets within the cell, whereas sublethal concentrations of triclosan specifically target the enoyl-acyl carrier protein (ACP) reductase FabI (28). A homolog to this ACP reductase in *Mycobacterium* spp., InhA, is targeted by the antimycobacterial agent isoniazid, and decreased susceptibility to triclosan correlates with decreased susceptibility to this antibiotic (27, 31). Also, both triclosan resistance and quinolone resistance map to genes encoding the same multidrug efflux pump in *P. aeruginosa* (7).

Several studies have found that exposure to QAC can alter antibiotic susceptibility (17, 19, 24, 25, 32). Sequential exposure of *S. enterica* serovar Typhimurium to a QAC biocide increased the MICs of tetracycline and ampicillin but did not affect the MIC for the biocide (17). When adapting different strains to QAC followed by increased MIC of the biocide, the MICs increased for several quinolones in *S. marcescens* (24), chloramphenicol in *E. coli* (19), and ciprofloxacin in *P. aeruginosa* (25). The MICs of gentamicin and kanamycin were increased for *L. monocytogenes* following adaption to the QAC biocide benzalkonium chloride (BC) (32). Other studies have not found any correlation between QAC tolerance and altered antibiotic MIC (1, 16). The different outcomes can be due to use of different bacteria, different QAC-containing biocides, different antibiotics tested, or different adaption/exposure techniques. To our knowledge, similar studies have not been conducted with *L. monocytogenes* with peroxy-based biocides. It has, however, been shown that hydrogen peroxide adaption can decrease susceptibility to tetracycline in *S. enterica* serovar Virchow (5).

TABLE 4. Antibiotic MICs for *L. monocytogenes* N53-1 gentamicin-resistant isolates pregrown without gentamicin

Strain	Triclosan exposure concn (μg/ml)	Isolate no.	MIC (μg/ml) ^a										
			GEN at subcultivation no.:					KAN	PEN	SPT	STR	TET	TOB
			1	2	3	4	5						
N53-1	0 (wt) ^d		5–10 ^b	5–10 ^b	10–20 ^b	10	10	16–32 ^b	0.5–1 ^b	128–256 ^b	20–40 ^b	2	4–8 ^b
	4	2-2	40–80 ^b	80	80	40–80 ^b	80	128	0.5	128	160	2	32
	1	1-1	80	80	80	80	80	128	0.5	128	160	1	64
		2-1	80	80	80	20	40	256	0.5	128	160	1	64
	0 (control)	1-1	10 ^c	5–10 ^b	5–10 ^b	5–10 ^b	5–10 ^b	32	0.5	128	80	2	8
4446	0 (wt)		20 ^c	ND	ND	ND	ND	16–32 ^b	0.25–0.5 ^b	128	40–80 ^b	2	16
	4	1	40	ND	ND	ND	ND	128	0.25	128	80	2	64
	1	1	80	ND	ND	ND	ND	128	0.25	256	80	0.25	>64

^a The MIC for gentamicin (GEN) was determined after each of five subcultivations on BHI agar without gentamicin. The MIC was determined after 20 to 22 h at 37°C. KAN, kanamycin; PEN, penicillin G; SPT, spectinomycin; STR, streptomycin; TET, tetracycline; TOB, tobramycin.

^b Ranges representing MICs differing by 2-fold when determined.

^c Medians of determined MICs are shown.

^d wt, wild type.

In the present study, exposure to triclosan at sublethal concentrations resulted in an increased gentamicin resistance frequency for eight *L. monocytogenes* strains independent of lineage, serotype, or whether the strains were persistent or presumed nonpersistent. Hence, the persistent strains were not, as we hypothesized, affected differently by the biocide compared to the presumed nonpersistent strains. We assume that *L. monocytogenes* strains can encounter triclosan in sublethal concentrations in their natural habitat due to the wide distribution and stability of the compound, and hence, one might speculate that *L. monocytogenes* can be exposed to concentrations affecting gentamicin susceptibility.

Gentamicin-resistant isolates also emerged from the control cultures for five of the eight *L. monocytogenes* strains tested, but with a lower frequency and not consistently throughout the four consecutive cultures. The instability of gentamicin MIC in an N53-1 isolate selected from the control and its highly decreased growth rate could indicate that the resistance mechanism is different from the one in the triclosan-exposed isolates.

The MICs for triclosan were similar in the wild-type and the gentamicin-resistant isolates; hence, triclosan exposure did not change susceptibility to triclosan but to aminoglycosides. This is, to the best of our knowledge, the first time that triclosan exposure has been linked to changed antibiotic susceptibility

while triclosan susceptibility was unaltered. Triclosan exposure altered both triclosan susceptibility as well as aminoglycoside susceptibility in *S. aureus* (36); however, such a connection was not seen in *E. coli*, *P. aeruginosa*, or *S. enterica* (5, 7, 17). Triclosan exposure selected for the formation of *S. aureus* small-colony variants that had up to a 10-fold increase in MIC for gentamicin compared to the wild type (36). In contrast to the present study, this was paralleled by a decrease in triclosan susceptibility (24- to 60-fold) (36).

Gentamicin-resistant *L. monocytogenes* strains were also resistant to kanamycin, streptomycin, and tobramycin, which all are aminoglycosides. Aminoglycoside resistance can be caused by multidrug efflux pumps (23); however, we found this mechanism unlikely, as susceptibility to triclosan and nonaminoglycoside antibiotics was not affected. Aminoglycoside resistance is often caused by aminoglycoside-modifying enzymes that can be plasmid or transposon encoded (37) or by a 16S rRNA methylase that alters the antibiotic's target site (9). However, such resistance would require presence of the genes encoding the enzymes in the eight wild-type *L. monocytogenes* strains, which could then, theoretically, be induced by triclosan exposure. However, if these genes were present in the wild-type strains, one would expect them to have a MIC of gentamicin similar to that for the tolerant isolates, which was not the case (Table 3).

Gentamicin, kanamycin, and tobramycin are all 4,6-disubstituted aminoglycosides, while streptomycin is classified as an aminoglycoside. These all interact with specific nucleotides within the 1,400- to 1,500-bp region in the 16S rRNA A site (12, 15, 29). Gentamicin and kanamycin bind to A1408 and G1494 in 16S rRNA (29), and tobramycin binds to an RNA aptamer (15), structurally similar to the A site of 16S rRNA, while streptomycin binds to bases 1394 to 1415 in 16S rRNA (12). Hence, mutations within the 3' end of 16S rRNA could cause the resistance in the triclosan-exposed isolates. No mutations, however, were found within this region in the selected resistant *L. monocytogenes* N53-1 isolates. During amplification of parts of the 16S rRNA-encoding regions, two primer sets that amplified the first four alleles or the last two alleles in EGD-e, respectively, were used. Further differentia-

TABLE 5. Generation time for *L. monocytogenes* wild-type and gentamicin-resistant N53-1 isolates when cultured at 37°C with and without gentamicin

Triclosan exposure concn (μg/ml)	Isolate no.	Generation time (min) ^a	
		–GEN	+GEN
0 (wt) ^b		37	NG
4	2-2	56	90
1	1-1	42	60
	2-1	41	49
0 (control)	1-1	74.5 ± 6.4	121 ± 11.3

^a Generation times were determined in single experiments, except from isolate no. 1-1 selected from 0 μg/ml triclosan (control), where two independent experiments were conducted. –GEN, without gentamicin; +GEN, with gentamicin; NG, no growth.

^b wt, wild type.

tion between alleles was not possible as the nucleotide sequences within and downstream of the genes are very similar. Hence, the affinity between the primer sets and the alleles can cause a bias, amplifying only a selection of alleles. Consequently, potential mutations in nonamplified alleles would not be identified. In addition, as several alleles were amplified using the same primer set, mutations within a single allele could be concealed by potentially nonmutated DNA in other alleles. Hence, we cannot exclude that mutations within a single allele have occurred. Cloning of each allele separately could clarify if mutations within single alleles had caused the found resistances.

How triclosan can induce changed susceptibility to aminoglycosides without affecting triclosan susceptibility is puzzling and should be studied further. Aslangul et al. (3) adapted *Enterococcus faecalis* to gentamicin, which resulted in a similar resistance phenotype to several aminoglycosides, as in the present study. Gentamicin-resistant *E. faecalis* isolates were resistant to kanamycin, streptomycin, and tobramycin, while spectinomycin susceptibility was not affected (3). All possible aminoglycoside resistance mechanisms were excluded, and impaired drug permeability was suggested as the cause, since gentamicin uptake was decreased in the isolates (3). Also, *E. coli* mutants with deficient aerobic transport had increased MICs for different aminoglycosides, but not spectinomycin, and decreased uptake of streptomycin and gentamicin (6). In *S. aureus*, small-colony variants can arise due to triclosan and gentamicin exposure. This phenotype has a reduced antimicrobial susceptibility, which in part is caused by breakdown in the bacterial electron transport chain (36). Further studies are required to elucidate if decreased drug uptake could cause the triclosan-induced resistance to aminoglycosides in *L. monocytogenes*.

We believe that this is the first study showing that sublethal concentrations of triclosan can induce resistance to aminoglycosides in the human pathogen *L. monocytogenes*. This is alarming since gentamicin is part of the common clinical regimen used to treat listeriosis, and further studies are needed to elucidate the precise causal relationship between triclosan exposure and gentamicin resistance.

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