Triclosan-Induced Aminoglycoside-Tolerant *Listeria monocytogenes* Isolates Can Appear as Small-Colony Variants

Vicky G. Kastbjerg,^{a*} Line Hein-Kristensen,^a Lone Gram^b

National Food Institute, Technical University of Denmark, Kongens Lyngby, Denmark^a; Department of System Biology, Technical University of Denmark, Kongens Lyngby, Denmark^b

Exposure of the human food-borne pathogen *Listeria monocytogenes* to sublethal concentrations of triclosan can cause resistance to several aminoglycosides. Aminoglycoside-resistant isolates exhibit two colony morphologies: normal-size and pinpoint colonies. The purposes of the present study were to characterize the small colonies of *L. monocytogenes* and to determine if specific genetic changes could explain the triclosan-induced aminoglycoside resistance in both pinpoint and normal-size isolates. Isolates from the pinpoint colonies grew poorly under aerated conditions, but growth was restored by addition of antibiotics. Pinpoint isolates had decreased hemolytic activity under stagnant conditions and a changed spectrum of carbohydrate utilization compared to the wild type and isolates from normal-size colonies. Genome sequence comparison revealed that all seven pinpoint isolates had a mutation in a heme gene, and addition of heme caused the pinpoint isolates to revert to normal colony size. Triclosan-induced gentamicin-resistant isolates had mutations in several different genes, and it cannot be directly concluded how the different mutations caused gentamicin resistance. However, since many of the mutations affected proteins involved in respiration, it seems likely that the mutations affected the active transport of the antibiotic and thereby caused resistance by decreasing the amount of aminoglycoside that enters the bacterial cell. Our study emphasizes that triclosan likely has more targets than just *fabI* and that exposure to triclosan can cause resistance to antibiotics that enters the cell via active transport. Further studies are needed to elucidate if *L. monocytogenes* pinpoint isolates could have any clinical impact, e.g., in persistent infections.

Listeria monocytogenes is a food-borne human pathogen that can cause the fatal infection listeriosis. The disease is commonly treated with either of the antibiotics ampicillin and penicillin G in combination with an aminoglycoside, usually gentamicin, since a synergistic effect of these antibiotics has been observed *in vitro* as well as on extracellular, multiplying *Listeria* (1, 2).

Generally, antibiotic resistance is uncommon in *L. monocytogenes*, but we have previously demonstrated that short-term exposure of the organism to sublethal triclosan concentrations (1 to 4 µg/ml) can cause resistance to several aminoglycosides with an inheritable increase in MIC of 4- to 8-fold (3). Notably, the aminoglycoside-resistant isolates were as sensitive to triclosan as the wild type (3). In the present study, we set out to determine the genetic causes of this resistance. We noticed that the resistant isolates appeared with two colony morphologies. Some isolates were of wild-type colony size (1.5 to 2.5 mm in diameter), whereas others grew as pinpoint colonies (<1 mm in diameter). Hence, we hypothesized that the latter could be a type of small-colony variant (SCV).

The so-called SCVs have been described for several bacteria, including *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Salmonella* serovars, *Vibrio cholerae*, *Shigella* spp., *Staphylococcus* spp., *Escherichia coli*, *Lactobacillus acidophilus*, and *Serratia marcescens* (reviewed in reference 4). SCVs constitute a slow-growing subpopulation of bacteria that form colonies nearly 1/10 the size of the colonies associated with wild-type bacteria, and they display distinctive phenotypic and pathogenic characteristics (4). The most extensively studied and well-described SCVs are those of *Staphylococcus aureus*. This is likely due to their clinical impact, as several studies have established an association between the recovery of *S. aureus* SCVs and persistent and relapsing infections (5, 6, 7). Two types of *S. aureus* SCVs are typically isolated: those that have an interrupted electron transport chain (ETC) and those that

are thymidine auxotrophic. ETC-deficient *S. aureus* bacteria can further be divided into those with mutations affecting biosynthesis of menadione or hemin (4). These are characterized by low growth rate, lack of pigmentation, decreased hemolytic activity, reduced spectrum of carbohydrate utilization, and increased resistance to aminoglycosides (8). *In vitro*, SCVs emerge in the presence of aminoglycosides, e.g., gentamicin (9), and interestingly triclosan also selects for *S. aureus* colonies of the SCV phenotype (10, 11).

Small colonies of *L. monocytogenes* have to our knowledge been described only for an *in vitro*-generated *perR* mutant of *L. monocytogenes* (12) and not for spontaneous generation in a selective environment *in vitro*. However, the existence of such an antibiotic-resistant state could be important for its persistence in the host and have consequences for antibiotic treatment of listeriosis.

The purposes of the present study were to characterize the small colonies of *L. monocytogenes* and to determine if specific genetic changes could explain the triclosan-induced aminoglycoside resistance and hence unravel the tolerance mechanism.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. *Listeria monocytogenes* strain N53-1 was grown in four successive overnight cultures with subin-

Received 18 October 2013 Returned for modification 31 December 2013

Accepted 10 March 2014

Published ahead of print 17 March 2014

Address correspondence to Lone Gram, gram@bio.dtu.dk.

* Present address: Vicky G. Kastbjerg, Novo Nordisk A/S, Copenhagen, Denmark.

Copyright © 2014, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AAC.02266-13

TABLE 1 Colony morphology and MICs of gentamicin for the *L. monocytogenes* N53-1 isolates used in the present study pregrown on BHI without or with gentamicin (16 µg/ml) and hemin (5 µg/ml)

Isolate	Triclosan concn (µg/ml) ^c	Colony morphology ^d	Selected after ^e	MIC (µg/ml) for isolates pregrown ^{a,b} :		
				Without GEN	With GEN	With heme
N53-1		N		2.5 (2.5)	ND	2.5 (1.88)
E34 ^f	4	N	96 h	40 (40)	80 (60)	40 (40)
105	4	N	96 h + 24 h	10 (10)	80 (80)	5 (7.5)
10	4	PP	96 h + 24 h	40 (40)	40 (40)	40 (40)
118	4	PP	96 h + 24 h	80 (60)	80 (60)	40 (40)
E38 ^f	2	N	96 h	40 (30)	40 (40)	10 (7.5)
13	2	N + PP	96 h + 24 h	40 (30)	80 (60)	80 (40)
20	2	PP	96 h + 24 h	40 (40)	40 (40)	40 (40)
21	2	PP	96 h + 24 h	40 (40)	40 (40)	40 (40)
28	1	N + PP	96 h + 24 h	80 (80)	80 (40)	40 (30)
69	1	N	96 h + 48 h	40 (30)	80 (60)	80 (60)
E18 ^f	1	PP	96 h	40 (40)	80 (80)	40 (40)
E41 ^f	1	PP	96 h	80 (60)	80 (80)	40 (40)
33	1	PP	96 h + 24 h	80 (80)	80 (80)	80 (60)
E22 ^f	0 (control)	N	96 h	2.5 (2.5)	40 (40)	20 (20)
37	0 (control)	N	96 h + 24 h	5 (5)	40 (40)	2.5 (2.5)

^a MIC was determined after 24 h at 37°C. All isolates were tested in duplicates in at least two biological replicates. GEN, gentamicin.^b Mode MIC. Median values are given in parentheses. ND, not determined.^c The triclosan concentration is the one that the isolate had been exposed to before selection on gentamicin in the study of Christensen et al. (3).^d The isolates grow as normal-size colonies (N; 1.5 to 2.5 mm in diameter) or as pinpoint colonies (PP) or as a mixture of those two.^e Isolates selected after 96 h of growth are from the study of Christensen et al. (3). Cultures from that study were reinoculated in BHI with triclosan and selected on BHI with gentamicin after 24 h and 48 h of growth.^f The isolates were also included in the work of Christensen et al. (3) with the following designations: E34, (4) 2-2; E18, (1) 1-1; E41, (1) 2-1; and E22, (0) 1-1.

hibitory triclosan concentrations (3). After overnight exposure, the populations were plated on gentamicin-containing agar, and isolates with enhanced MICs were selected (3). Fifteen aminoglycoside-tolerant *L. monocytogenes* N53-1 isolates were selected and compared to the wild type, which is a persistent subtype frequently occurring in the fish processing industry (13) (Table 1). Five of 15 isolates were included in the study by Christensen et al. (3), and 10 isolates were selected for this study from the triclosan-exposed populations as follows. Triclosan-exposed populations (42 µl) were inoculated in brain heart infusion (BHI) broth (9.8 ml) (Oxoid; CM1135) with the concentration of triclosan that they were originally isolated from (0, 1, 2, and 4 µg/ml, respectively). After growth for 24 h at 37°C with shaking (250 rpm), the cultures were diluted to 10⁴ CFU/ml and regrown with triclosan. In total, two consecutive 24-h cultures were done for each population. To select for gentamicin-tolerant isolates in the populations, 100 µl of outgrown culture was streaked on BHI agar (BHI broth supplemented with 1.5% agar (VWR; 20768.292) with 16 µg/ml of gentamicin (Sigma-Aldrich; G3632) (2× the MIC for the wild type) and incubated for 48 h at 37°C. Resistant isolates were cultured at 37°C in BHI broth supplemented with 5.3 or 2.7 µg/ml of gentamicin and stored in 15% glycerol at −80°C. Two pinpoint and two normal-size colonies (1.5 to 2.5 mm in diameter) from each triclosan-exposed population were chosen when possible (Table 1). The isolates were cultivated on BHI agar for 2 days at 37°C. The pinpoint phenotype was stable, and the isolates did not revert to normal size either during long incubation on BHI agar (10 days at 37°C) or when restreaked 10 times on BHI agar.

MICs of gentamicin and triclosan in liquid media. The gentamicin and triclosan MICs of the wild type and 15 resistant isolates were determined by a broth microdilution method as described by Christensen et al. (3), with the modification that microtiter plates were incubated for 24 h at 37°C. The MIC was determined for *L. monocytogenes* grown on BHI agar with or without 16 µg/ml of gentamicin and on BHI agar with or without 5 µg/ml of hemin in duplicate in two biological replicates.

Planktonic growth measurements. The wild-type and resistant isolates of *L. monocytogenes* were suspended in 0.9% saline to an optical density at 546 nm (OD₅₄₆) of 0.13 using a Novaspec III (Amersham Biosciences) corresponding to a cell density of 10⁸ CFU/ml. This stock was

diluted and inoculated to a level of 10⁵ CFU/ml in 5 ml of BHI in a 50-ml tube. Cultures were incubated at 37°C with shaking (300 rpm) or under stagnant conditions for 24 h. Growth was measured as OD₆₀₀ and as number of CFU/ml on BHI agar. The cultures of pinpoint isolates reached an OD₆₀₀ of approximately 0.1 after 24 h under shaking conditions, but the level of CFU was very low (see Results). Under stagnant conditions, the culture reached an OD₆₀₀ of 1 and a level of approximately 10⁹ CFU/ml. We therefore followed the growth of two pinpoint isolates, E41 and E18, and the wild type, N53-1 (in biological replicates), in more detail. To determine when the CFU declined, OD₆₀₀ and number of CFU were determined after 7, 8.5, 10, 12, 14, 16, 18, 20, 22, and 24 h of growth. To determine if any specific degree of aeration caused a decrease in the survival of the pinpoint isolates, pinpoint isolates E18 and E41, normal-size isolate E34, and the wild type were grown in BHI at 37°C under four different shaking conditions: 0, 100, 200, and 300 rpm.

Killing experiments with gentamicin and ampicillin. Survival of the wild type and *L. monocytogenes* mutants during exposure to 10, 20, 40, or 80 µg/ml of gentamicin was determined. Suspended cultures were prepared in saline and inoculated as described above. The number of CFU was determined after 7 and 24 h (37°C and 300 rpm) on BHI agar. To elucidate if ampicillin acted synergistically with gentamicin, four gentamicin-resistant isolates (two pinpoint [E18 and E41] and two normal size [E34 and 69]) and the wild type, N53-1, were exposed to a final concentration of 2 µg/ml of ampicillin either alone or in combination with 10 or 20 µg/ml of gentamicin. The number of CFU was determined after 3, 6, and 24 h of growth (37°C and 300 rpm). The killing experiments were performed in two biological replicates.

Growth experiments with catalase, hemin, menadione, and menaquinone. To determine the factors that caused the decrease in variable cell count of the isolates from pinpoint colonies during aerated growth, and to determine if the *L. monocytogenes* pinpoint isolates were similar to the small-colony variants described for *Staphylococcus aureus*, growth experiments with oxygen-protective compounds and vitamin Ks were performed. Growth of the *L. monocytogenes* wild type and mutants was determined in BHI broth as described earlier under both shaking and stagnant conditions and on cultures streaked on BHI agar. The BHI broth

was supplemented with catalase, hemin, menadione, and menaquinone as follows: BHI broth was supplemented with 170 U/ml of sterile filtered catalase (Fluka; 60634) and BHI agar with 1,700 or 170 U/ml. BHI broth was supplemented with hemin (Sigma; 51280; dissolved in 0.05 M NaOH) to a final concentration of 1 µg/ml and BHI agar with 5 µg/ml of hemin. BHI broth was supplemented with menadione (Sigma; M 5625; dissolved in 99% ethyl alcohol [EtOH]) or menaquinone (Supelco; 47774; dissolved in 99% EtOH) to final concentrations of 1, 2, 5, 10, and 20 µg/ml and BHI agar to final concentrations of 5, 10, 20, 40, 80, and 160 µg/ml.

Biolog carbon substrate utilization assay. The *L. monocytogenes* wild type and mutants were pregrown on BHI agar (37°C, 48 h), and colony mass of *L. monocytogenes* was dissolved in 0.85% saline to an OD₆₀₀ of 0.3 (approximately 3×10^8 CFU/ml). Suspensions of 150 µl were dispensed into wells of a GP2 microplate (Biolog, CA). Plates were incubated at 37°C, and color development was determined visually after 4, 24, and 48 h of incubation. Each strain was assayed once.

Determination of hemolytic (LLO) activity. Listeriolysin O (LLO) activity was determined in culture supernatants of the *L. monocytogenes* wild type and mutants prepared in 0.9% saline and grown for 14 h (37°C) with or without shaking (300 rpm). Cultures were adjusted to an OD₆₀₀ of 1.0 and assayed by manual quantification of cell lysis activity in serial dilutions of supernatants using calf blood as described by Rodriguez et al. (14) and Leimeister-Wächter and Chakraborty (15). The plates were incubated at 37°C for 45 min, and the hemolytic activity in each well was scored as follows: 3, strong hemolysis; 2, moderate hemolysis; 1, weak hemolysis; 0.5, questionable hemolysis; and 0, no hemolysis. The hemolytic activity was summed and related to the OD₆₀₀ of each strain. The assay was carried out in two independent replicates.

Whole-genome sequencing. We sequenced the genomic DNA from the wild type and the 15 mutants to determine if this would reveal their mechanism for tolerance to gentamicin and explain the differences in colony morphology and phenotype. Genomic DNA was isolated from *L. monocytogenes* using the FastDNA kit (MP Biomedicals; 116540-400) as described by Holch et al. (16). Samples were RNase treated (Qiagen; 158922) before quantification, and quality was analyzed using 1% agarose gel electrophoresis, a NanoDrop spectrophotometer (Saveen Werner, Sweden), and a Qubit 2.0 analyzer (Invitrogen, United Kingdom). Libraries of 500 bp were used for 100-bp paired-end sequencing of genomes using Illumina sequencing technology on a HiSeq2000 with a minimum coverage of 100 (Beijing Genomics Institute, Hong Kong, China). *L. monocytogenes* N53-1 was *de novo* assembled into contigs using CLCbio Genomics Workbench (Aarhus, Denmark), resulting in 36 contigs comprising 3.1 Mbp. Using this procedure, the 15 triclosan-exposed isolates were mapped with the 39 contigs as a reference with a minimum coverage of 100, and single-nucleotide polymorphisms (SNPs) and deletion-insertion polymorphisms (DIPs) with a frequency of 80% were detected. Only mutations causing an amino acid change are included in the results.

Statistical analysis. Bacterial counts from growth assays were log transformed before calculating mean values and standards deviations. Means of hemolytic activity for isolates grown under stagnant and shaking conditions were statistically compared by using Student's *t* test with Welch's correction. Means of hemolytic activity for normal-size and pinpoint isolates (mixed populations 13 and 28 not included) grown under shaking or stagnant conditions were statistically compared by using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test. All statistical analyses were performed with GraphPad Prism statistical software.

Nucleotide sequence accession numbers. The results of the whole-genome shotgun sequencing have been deposited in DDBJ/EMBL/GenBank under accession number [AXDU000000000](#). The version described in this paper is version [AXDU01000000](#).

RESULTS

Both pinpoint and normal-size colonies can be found in the triclosan-exposed populations. We selected a total of 15 gentami-

cin-resistant isolates of *L. monocytogenes* N53-1 from triclosan-exposed populations (3). From all populations, independent of the triclosan concentration, the gentamicin-resistant isolates appeared with two morphologies: pinpoint and normal-size colonies (1.5 to 2.5 mm in diameter). No pinpoint colonies appeared from the control cultures that had not been exposed to triclosan. Hence, the pinpoint morphology was related to the exposure to triclosan but not to a specific concentration. Two pinpoint and two normal-size colonies from each triclosan-exposed population were included in the present study (Table 1). Isolates with similar colony morphologies and exposed to the same triclosan concentration were picked from different populations to ensure that they were not clonal. We found that the isolates from pinpoint colonies were impaired in catalase production as determined after exposure to 3% H₂O₂.

Triclosan-exposed isolates have increased MICs to gentamicin but not to triclosan. The MICs were determined for *L. monocytogenes* N53-1 (wild type) and the 15 resistant mutants. Isolates pregrown with gentamicin had a 16- to 32-fold increase in gentamicin MIC compared to that of the wild type (40 to 80 µg/ml versus 2.5 µg/ml), and the level of resistance was independent of colony morphology (Table 1). The gentamicin MIC remained high also when the isolates were pregrown without gentamicin, except for the two isolates that had been selected from the control (non-triclosan-exposed) cultures (E22 and 37), for which the gentamicin MIC decreased to a level similar to that seen for the wild type. Also, for one of the isolates from normal-size colonies selected from 4 µg/ml of triclosan (isolate 105), the MIC decreased to 10 µg/ml (Table 1). The triclosan MIC was 4 µg/ml for all isolates, independent of exposure concentration or preculture conditions (data not shown). Hence, the gentamicin sensitivity was the same for all isolates independent of colony size. However, due to the different morphologies, we speculated that the resistance mechanism could be different in the two groups of isolates.

Isolates from pinpoint colonies die under shaking but not under stagnant conditions. The wild type and 15 mutants were grown under stagnant and shaking conditions. All isolates reached an OD₆₀₀ of 1.0 to 1.6 under stagnant conditions and a cell density of approximately 1×10^9 CFU/ml after 24 h, except for two isolates (13 and 28) that only reached an OD₆₀₀ of 0.3 and a cell density of approximately 1×10^8 CFU/ml (data not shown). Surprisingly, the cultures of the pinpoint isolates reached an OD₆₀₀ of 0.8 to 1.0, but the CFU/ml was only between 10^2 and 10^4 following 24 h of aerated growth (data not shown). No difference in cell morphology or cell number was observed when the cultures were studied by light phase-contrast microscopy (magnification of $\times 1,000$), indicating that bacterial cell death seen in the cultures of the pinpoint isolates was not a consequence of cell lysis.

The kinetics of changes in CFU for the cultures of pinpoint isolates during aerated growth were studied with two isolates, E41 and E18, and the wild type, N53-1. Under stagnant conditions, OD₆₀₀s and viable counts of all three isolates increased for 12 h and then stabilized in the stationary phase (Fig. 1A). Similarly, under shaking conditions, OD₆₀₀s increased for 12 h and stabilized in the stationary phase, whereas the viable counts of the cultures of pinpoint isolates declined after 14 h from 10^9 CFU/ml to 10^4 CFU/ml at 24 h (Fig. 1B).

We exposed isolates from two normal-size and pinpoint colonies to different shaking conditions and found that all isolates achieved similar cell numbers, 1×10^9 CFU/ml, after 24 h at 0 and

FIG 1 Growth of *L. monocytogenes* N53-1 (wild type) (■) and triclosan-exposed pinpoint isolates E18 (▲) and E41 (●) in BHI at 37°C under stagnant (A) and aerated (300 rpm) (B) conditions. Error bars show standard deviations ($n = 2$).

100 rpm. The cultures of normal-size isolates also reached a cell density of 1×10^9 CFU/ml after 24 h of growth at 200 and 300 rpm; however, the survival of the cultures of pinpoint isolates decreased markedly, to 10^3 to 10^4 CFU/ml, at these speeds (data not shown), indicating that the decrease in viability could be related to oxygen tension in the growth medium.

Addition of gentamicin increases survival of cultures from pinpoint isolates. As aeration influenced the survival of the isolates from pinpoint colonies, we also studied the survival of cultures from both pinpoint and normal-size colonies during growth with antibiotic. As expected, the survival of isolates from normal-size colonies decreased with increasing concentration of gentamicin (Fig. 2A). Interestingly, for the pinpoint isolates, addition of gentamicin increased the survival after 24 h of exposure (Fig. 2B). Typically, cultures of pinpoint isolates had low viable cell numbers (10^3 to 10^5 CFU/ml) after 24 h without gentamicin. However, the addition of 10 and 20 $\mu\text{g/ml}$ of gentamicin increased bacterial cell counts, whereas addition of higher concentrations of gentamicin (40 and 80 $\mu\text{g/liter}$) caused the bacterial survival cell number to decrease. Hence, there is a concentration window where addition of antibiotics can increase the survival of pinpoint isolates grown under aerated conditions.

There is still synergism between gentamicin and ampicillin. Preliminary killing experiments with ampicillin demonstrated that the antibiotic is bacteriostatic, and the killing effects on cultures of normal-sized and pinpoint isolates were similar at 1, 2, and 4 $\mu\text{g/ml}$ of ampicillin. After 24 h of ampicillin treatment, the bacterial numbers were from 2×10^3 to 4×10^4 at 1 $\mu\text{g/ml}$, from 5×10^3 to 5×10^4 at 2 $\mu\text{g/ml}$, and from 5×10^3 to 5×10^4 at 4 $\mu\text{g/ml}$ of ampicillin. We combined 2 $\mu\text{g/ml}$ of ampicillin, corresponding to $2 \times \text{MIC}$, with 10 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$ of gentamicin, corresponding to $0.25 \times$ and $0.5 \times \text{MIC}$ for the gentamicin-resistant isolates, respectively.

Gentamicin at 10 and 20 $\mu\text{g/ml}$ caused only a marginal reduction in cell counts for the normal-size isolates E34 and 69. However, the addition of 2 $\mu\text{g/ml}$ of ampicillin in combination with gentamicin decreased bacterial numbers markedly, from 10^9 CFU/ml to around 1 to 10^2 CFU/ml after 24 h (Fig. 3B and C). Gentamicin and ampicillin acted as bacteriostatic and not as bac-

FIG 2 Bacterial counts of *L. monocytogenes* N53-1 wild-type (■) and triclosan-exposed normal-size isolates (E34 [open squares], 105 [solid triangles], E38 [open triangles], 13 [open circles], 28 [solid circles], 69 [plus signs], E22 [minus signs], and 37 [open diamonds]) (A) and pinpoint isolates (10 [multiplication signs], 118 [long thick bars], 20 [open squares], 21 [solid circles], E18 [solid triangles], E41 [solid diamonds], and 33 [short thick bars]) (B) after 24 h of growth in BHI at 37°C and 300 rpm with gentamicin. Data are means of two biological replicates. Error bars show standard deviations ($n = 2$).

tericidal compounds against the pinpoint isolates (Fig. 3D and E). The largest difference in reduction of bacterial cells was found at 6 h, when addition of ampicillin caused an increased reduction, of approximately 2 logs, compared to that with addition of gentamicin alone. However, at 24 h, the synergistic effect of ampicillin was less pronounced for E41 and E18.

Isolates from pinpoint colonies survive growth under shaking conditions in the presence of hemin and catalase. As the pinpoint isolates lost culturability under aerated, but not under stagnant, conditions, we hypothesized that this was caused by enhanced sensitivity to oxygen concentrations. This could correspond to the catalase negativity of the pinpoint isolates. In *S. aureus*, the SCV phenotype is characterized by auxotrophy for hemin and menadione, and the SCV phenotype can be completely reversed by addition of these compounds (9, 17).

Menadione or menaquinone was added to BHI broth, but this did not increase survival of the pinpoint isolates after 24 h of growth with aeration, and the isolates grew as pinpoint isolates on BHI agar supplemented with menadione or menaquinone (data not shown).

In contrast, addition of 1 $\mu\text{g/ml}$ of hemin to BHI broth increased the survival of the pinpoint isolates in aerated cultures markedly (from 10^2 to 10^9 CFU/ml) compared to that in BHI without hemin, without affecting the survival of the normal-size isolates (Fig. 4A). Interestingly, the colony size of the pinpoint isolates increased when they were grown on BHI agar with 5 $\mu\text{g/ml}$ of hemin, to a diameter of 1.5 to 2.5 mm, corresponding to the normal size of *L. monocytogenes*. These isolates were catalase positive. However, when these hemin-grown colonies were re-streaked on BHI agar, they reverted to catalase-negative pinpoint colonies, indicating that hemin must be present in order for the isolates to grow as normal-size colonies. The addition of 5 $\mu\text{g/ml}$ of hemin to BHI agar did not cause any difference in the colony size of the normal-size isolates. We determined the MIC to gentamicin of the *L. monocytogenes* wild type and mutants pregrown on BHI agar with hemin and found that the MIC was similar to the MIC determined for the isolates pregrown without gentamicin, except for E38, for which it decreased from 40 to 10 $\mu\text{g/ml}$, and for E22, for which it increased from 2.5 to 20 $\mu\text{g/ml}$ (Table 1). The fact that the MICs are identical for pinpoint isolates exhibiting pinpoint colony size (on BHI without gentamicin) or normal colony size (on BHI with hemin) indicates that the growth as pinpoint colonies *per se* does not cause gentamicin resistance.

Also, addition of 170 U of catalase increased the survival of the

FIG 3 Counts of *L. monocytogenes* N53-1 (wild type) (A) and triclosan-exposed normal-size isolates E34 (B) and 69 (C) and pinpoint isolates E18 (D) and E41 (E) during 24 h of growth at 37°C and 300 rpm in BHI (▲), with 10 µg/ml of gentamicin (■), with 10 µg/ml of gentamicin plus 2 µg/ml of ampicillin (□), with 20 µg/ml of gentamicin (●), and with 20 µg/ml of gentamicin plus 2 µg/ml of ampicillin (○). The results are representative of two independent experiments.

cultures from pinpoint colonies under shaking conditions, as the cell number increased from 10^2 to 10^9 CFU/ml (Fig. 4B). However, addition of catalase to BHI agar did not cause an increase in colony diameter of the pinpoint isolates either with 170 U or with 1,700 U (data not shown).

Sugar metabolism of triclosan-exposed isolates is changed. A metabolism profile for 96 carbohydrates was performed in Biolog plates for the *L. monocytogenes* wild type and mutants. All utilized *N*-acetyl-D-glucosamine, D-fructose, α-D-glucose, D-mannose, pyruvate acid methyl ester, and glycerol (data not shown). However, the utilization of 22 carbohydrates was changed in the triclosan-exposed isolates compared to the wild type (Table 2). Most of the isolates could use dextrin, D-psicose, adenosine, inosine,

FIG 4 Survival of *L. monocytogenes* N53-1 triclosan-exposed isolates grown in BHI at 37°C and 300 rpm for 24 h (solid bars) or with addition of hemin (A) or catalase (B) (open bars). Normal-size isolates are N53-1, E34, 105, E38, 69, E22, and 37. Pinpoint isolates are 10, 118, 20, 21, E18, E41, and 33. Mixed isolates (normal and pinpoint) are 13 and 28. Data are means of two biological replicates. Error bars show standard deviations ($n = 2$).

and uridine. The normal-size isolates, including the wild type, utilized arabinose, D-cellobiose, β-methyl-D-glucoside, salicin, xylose, gentiobiose, and *N*-acetyl-β-D-mannosamine, whereas the pinpoint isolates did not. In contrast, most of the pinpoint isolates utilized L-lactic acid and pyruvic acid, whereas the normal-size ones did not. Interestingly, only the wild type and isolates 105, 37, and 22, which all have low MICs compared to the other isolates, could use α-cyclodextrin, β-cyclodextrin, amygdalin, maltotriose, and D-trehalose.

The hemolytic activity of pinpoint isolates is lower than that of normal-size isolates during stagnant growth conditions. We investigated if the listeriolysin O activity was decreased in the pinpoint isolates as seen for *S. aureus* SCVs. Overall, isolates grown under stagnant conditions had a significantly higher hemolytic activity than did isolates grown under shaking conditions ($P < 0.0001$) (Fig. 5). No significant difference in hemolytic activity was observed between normal-size and pinpoint isolates under shaking conditions ($P > 0.05$). However, under stagnant conditions, the normal-size isolates had significantly higher hemolytic activity than did the pinpoint isolates ($P < 0.05$).

All pinpoint isolates have a mutation in a heme gene. The genome of *L. monocytogenes* N53-1 was assembled by *de novo* assembly to 36 contigs with a total size of 3.1 Mbp and a GC content of 37.84%. Only SNPs and DIPs with a frequency of >80% that caused an amino acid change in an open reading frame were included in the further analysis. This gave 0 to 3 mutations for each isolate (Table 3). No mutations were detected in the control isolate (E22) that had been grown without triclosan. The other control isolate (isolate 37) had a DIP in ATP synthase subunits. Interestingly, all seven pinpoint isolates had a mutation in a heme gene, in either the ferrochelatase gene (*hemH*) or the glutamyl-tRNA reductase gene (*hemA*). These mutations were not present in the

TABLE 2 Utilization of carbohydrates by *L. monocytogenes* N53-1 (wild type) and 15 triclosan-exposed isolates^a

Compound(s)	Isolates which respired compound	Isolates which did not respire compound
α -Cyclodextrin, β -cyclodextrin, amygdalin, maltotriose, D-trehalose	N53-1 ^w , 105 ⁿ , 37 ⁿ , E22 ⁿ	E34 ⁿ , E38 ⁿ , 69 ⁿ , 13 ^m , 28 ^m , 20 ^{pp} , 21 ^{pp} , E18 ^{pp} , E41 ^{pp} , 33 ^{pp} , 10 ^{pp} , 118 ^{pp}
Arabin, D-cellobiose, β -methyl-D-glucoside, salicin, xylitol	N53-1 ^w , 105 ⁿ , 37 ⁿ , E22 ⁿ , E34 ⁿ , E38 ⁿ , 69 ⁿ , 13 ^m , 28 ^m	20 ^{pp} , 21 ^{pp} , E18 ^{pp} , E41 ^{pp} , 33 ^{pp} , 10 ^{pp} , 118 ^{pp}
N-Acetyl- β -D-mannosamine	N53-1 ^w , 105 ⁿ , 37 ⁿ , E22 ⁿ , E34 ⁿ , E38 ⁿ , 69 ⁿ , 13 ^m	20 ^{pp} , 21 ^{pp} , E18 ^{pp} , E41 ^{pp} , 33 ^{pp} , 10 ^{pp} , 118 ^{pp} 28 ^m
Gentibiose	N53-1 ^w , 105 ⁿ , 37 ⁿ , E22 ⁿ , E34 ⁿ , E38 ⁿ , 69 ⁿ , 28 ^m	20 ^{pp} , 21 ^{pp} , E18 ^{pp} , E41 ^{pp} , 33 ^{pp} , 10 ^{pp} , 118 ^{pp} 13 ^m
Dextrin, adenosine, inosine	N53-1 ^w , 105 ⁿ , 37 ⁿ , E22 ⁿ , E38 ⁿ , 69 ⁿ , 20 ^{pp} , 21 ^{pp} , E18 ^{pp} , E41 ^{pp} , 33 ^{pp} , 10 ^{pp} , 118 ^{pp}	E34 ⁿ , 28 ^m , 13 ^m
D-Psicose, uridine	N53-1 ^w , 105 ⁿ , 37 ⁿ , E22 ⁿ , E34 ⁿ , E38 ⁿ , 69 ⁿ , 20 ^{pp} , 21 ^{pp} , E18 ^{pp} , E41 ^{pp} , 33 ^{pp} , 10 ^{pp} , 118 ^{pp}	28 ^m , 13 ^m
L-Lactic acid	20 ^{pp} , E41 ^{pp} , 10 ^{pp} , 118 ^{pp}	N53-1 ^w , 105 ⁿ , 37 ⁿ , E22 ⁿ , E34 ⁿ , E38 ⁿ , 69 ⁿ , 28 ^m , 13 ^m 21 ^{pp} , E18 ^{pp} , 33 ^{pp}
Pyruvic acid	105 ⁿ E22 ⁿ E38 ⁿ , 20 ^{pp} , 21 ^{pp} , E18 ^{pp} , E41 ^{pp} , 33 ^{pp} , 10 ^{pp} , 118 ^{pp}	N53-1 ^w , 37 ⁿ , E34 ⁿ , 69 ⁿ , 28 ^m , 13 ^m
Maltose, AMP, UMP	N53-1 ^w , 105 ⁿ , 37 ⁿ , E22 ⁿ , E38 ⁿ , 69 ⁿ	E34 ⁿ 28 ^m , 13 ^m 20 ^{pp} , 21 ^{pp} , E18 ^{pp} , E41 ^{pp} , 33 ^{pp} , 10 ^{pp} , 118 ^{pp}

^a Only carbohydrates with differences between isolates are shown. Respiration was determined in Biolog plates after 24 h at 37°C. Superscripts indicate morphology as follows: w, wild type; n, normal size; pp, pinpoint; m, mixed (normal plus pinpoint).

normal-size resistant isolates. Some mutations were present in both normal-size and pinpoint isolates: translation elongation factor G (*fusA*) (3 isolates), geranyltransferase (4 isolates), and different subunits from the ATP synthase (*atpA*, *atpD*, and *atpG*) (8 isolates). Two mutations were present only in some of the pinpoint isolates: TPR repeat-containing protein YrrB (*yrrB*) (1 isolate) and *N*-acetylglucosamine-6-phosphate deacetylase (1 isolate). Three mutations were detected only in some of the normal-size isolates, namely, in an ABC transporter CydDC (*cydD* and *cydC*) and the RNA polymerase sigma B factor (*rsbW*) (1 isolate) and a probable diguanylate cyclase, YcdT (*ycdT*) (1 isolate). There was no correlation between mutations detected and the concentration of triclosan to which an isolated had been exposed.

DISCUSSION

We have previously demonstrated that the food-borne human pathogen *L. monocytogenes* can develop resistance to gentamicin and other aminoglycosides following exposure to the widely used biocide triclosan (3). The gentamicin-resistant isolates exhibit two colony morphologies: normal-size and pinpoint colonies. Similarly, Latimer et al. (10) and Seaman et al. (11) found that triclosan can select for *S. aureus* colonies showing the characteristic SCV

phenotype. Seaman et al. (11) found, as did we, that isolates from the SCVs had reduced susceptibility to gentamicin, but they also had a low-level triclosan resistance in the study by Seaman et al. which we did not observe in this study. However, the lack of resistance to triclosan in isolates from pinpoint colonies could be due to the intrinsically higher MIC of *L. monocytogenes* to triclosan than that of *S. aureus* (18).

The *L. monocytogenes* pinpoint isolates did not survive well during aerated conditions, but addition of catalase and hemin increased growth and culturability. The catalase enzyme protects bacteria against oxygen stress, and *L. monocytogenes* is a catalase-positive bacterium. However, the pinpoint isolates were catalase negative, indicating that they produced no or only very low levels of catalase, and therefore, they were not able to protect themselves against oxygen. Hence, it seems likely that the addition of catalase to the growth media exerts the oxygen protection that the bacteria cannot perform themselves. The catalase-negative phenotype did not cause the small colony phenotype *per se*, as the addition did not support reversion to normal-size colonies. In contrast, the addition of hemin increased growth of the pinpoint isolates under shaking conditions and caused the bacteria to grow as normal-size colonies. This could be because the addition of hemin counteracts the mutation in heme-containing proteins that were detected in the pinpoint isolates. Similarly, a *hemB* mutant of *S. aureus* supplemented with hemin at a concentration of 1 μ g/ml showed a growth curve very similar to that of the parent strain (determined as OD measurements) (19). Pregrowth with hemin did not increase the sensitivity of our isolates to aminoglycosides as was seen for the *hemB* mutant of *S. aureus* (19). However, hemin was not present during exposure to the antibiotics in our study, as it was in the work of von Eiff et al. (19).

Addition of gentamicin in particular concentrations caused the pinpoint isolates to survive better during aerated culture, indicating that addition of antibiotics can abolish the oxygen stress. We have recently found that sublethal concentrations of gentamicin cause upregulation of the gene *lmo1634* in *L. monocytogenes* (G. M. Knudsen, Y. Y. Ng, and L. Gram, unpublished data). This could explain the increased survival of the oxygen-sensitive isolates, since *lmo1634* is homologous to the alcohol dehydrogenase

FIG 5 Hemolytic activities of *L. monocytogenes* N53-1 triclosan-exposed isolates grown in BHI at 37°C for 24 h under shaking (300 rpm) (solid bars) or stagnant (open bars) conditions. Normal-size isolates are N53-1, E34, 105, E38, 69, E22, and 37. Pinpoint isolates are 10, 118, 20, 21, E18, E41, and 33. Mixed isolates (normal and pinpoint) are 13 and 28. Data are means of two biological replicates. Error bars shows standard deviations ($n = 2$).

TABLE 3 Distribution of SNPs and DIPs that caused an amino acid change and had a frequency above 80% in the 15 gentamicin-tolerant *L. monocytogenes* N53-1 isolates

Isolate	Colony morphology ^a	Variation type	Amino acid change(s) ^b	Gene product
E34	N	SNP	Glu39Lys	Geranyltranstransferase
		SNP	Glu488Leu	ATP synthase F ₁ alpha subunit (<i>atpA</i>)
105	N	SNP	Ser63Gly	Probable diguanylate cyclase YcdT (<i>ycdT</i>) (16)
		DIP	Asn232delinsMetHis, Leu58delinsCysMet	ATP synthase F ₁ alpha subunit (<i>atpA</i>) and beta subunit (<i>atpD</i>)
E38	N	SNP	Thr20Arg	Geranyltranstransferase
		SNP	Met285Lys	ATP synthase F ₁ beta subunit (<i>atpD</i>)
69	N	SNP	Gly505Ser	Translation elongation factor G (<i>fusA</i>)
		DIP	Ala278fs	ABC transporter CydDC (<i>cydDC</i>)
		DIP	Glu31fs	RNA polymerase SigB factor (<i>rsbW</i>)
37	N	DIP	His219fs, Cys71fs	ATP synthase F ₁ gamma subunit (<i>atpG</i>) beta subunit (<i>atpD</i>)
		DIP	Ala220fs, Ala70fs	ATP synthase F ₁ gamma subunit (<i>atpG</i>) and beta subunit (<i>atpD</i>)
10	PP	SNP	His182Tyr	Ferrochelatase (<i>hemH</i>)
		SNP	Arg127Cys	ATP synthase F ₁ alpha subunit (<i>atpA</i>)
118	PP	SNP	Gly542Val	Translation elongation factor G (<i>fusA</i>)
		DIP	Ala98fs	Ferrochelatase (<i>hemH</i>)
20	PP	SNP	Gln190 ^c	Ferrochelatase (<i>hemH</i>)
		SNP	Leu301Ile	Hypothetical protein
		SNP	Arg248Ser, Arg42Leu	ATP synthase F ₁ gamma subunit (<i>atpG</i>) and beta subunit (<i>atpD</i>)
21	PP	SNP	Gln114 ^c	TPR repeat-containing protein YrrB (<i>yrrB</i>) (16)
		SNP	Gln190 ^c	Ferrochelatase (<i>hemH</i>)
E18	PP	SNP	Gln225 ^c	Glutamyl-tRNA reductase (<i>hemA</i>)
		SNP	Pro145Leu	ATP synthase F ₁ alpha subunit (<i>atpA</i>)
E41	PP	SNP	Ala578Val	Translation elongation factor G (<i>fusA</i>)
		SNP	Gln225 ^c	Glutamyl-tRNA reductase (<i>hemA</i>)
		DIP	Gly115fs	N-Acetylglucosamine-6-phosphate deacetylase
33	PP	SNP	Gln225 ^c	Glutamyl-tRNA reductase (<i>hemA</i>)
		SNP	Asp312Asn	ATP synthase F ₁ beta subunit (<i>atpD</i>)
13	N + PP	SNP	Thr20Arg	Geranyltranstransferase
28	N + PP	SNP	Lys158 ^c	Geranyltranstransferase

^a N, normal colony size (1.5 to 2.5 mm in diameter); PP, pinpoint colonies.
^b fs, frameshift mutation.
^c The mutation causes a stop codon.

gene, *adhE*, in *E. coli*, which during aerated growth plays a role in protection against oxidative stress (20). The gentamicin protection indicates that the pinpoint isolates do have an advantage during antibiotic stress regardless of the growth conditions. A synergistic effect has been demonstrated between gentamicin and ampicillin in the treatment of listeriosis (1, 2), and this effect was also demonstrated in this study for the pinpoint isolates. Hence, the use of the two antibiotics in combination seems to be essential in order to eliminate the *L. monocytogenes* pinpoint isolates.

The utilization of 22 carbohydrates was changed in the triclosan-exposed isolates compared to that of the wild type (Table 2). Similarly, the carbohydrate metabolism was changed in a *hemB* mutant of *S. aureus*, studied with phenotype microarray profiling (21). As with our pinpoint isolates, the *hemB* mutant of *S. aureus* had also lost the ability to use N-acetyl-β-D-mannosamine and β-methyl-D-glucoside. Also, the *hemB* SCV had lost the ability to utilize D-psicose and uridine, which we did not see for the pinpoint isolates of *L. monocytogenes*. In contrast, our pinpoint isolates had lost their ability to utilize maltose, which was not seen for *hemB* SCV.

The *L. monocytogenes* pinpoint isolates had significantly decreased hemolytic activity when pregrown under stagnant conditions. Similarly, *S. aureus* SCVs are also generally characterized by impaired hemolytic activity, and this also includes triclosan-ex-

posed *S. aureus* SCVs (10). Hemolytic activity is one of the virulence factors in *L. monocytogenes*, and one might speculate that pinpoint isolates of *L. monocytogenes* could be less virulent than wild-type *L. monocytogenes*. This was demonstrated for an *S. aureus* SCV strain developed by sublethal exposure to triclosan that had impaired hemolytic activity, was defective in biofilm formation and DNase activity, and displayed significantly attenuated virulence *in vivo* in *Galleria mellonella* (10). Also, clinical *S. aureus* SCVs as well as *hemB*- and *menD*-deficient mutants of *S. aureus* have been found to be greatly reduced in virulence in the *Caenorhabditis elegans* infection model (22). In contrast, several reports have demonstrated that *S. aureus* SCVs are linked with persistent infections (5, 6, 7), and a generated *hemB* mutant causing *S. aureus* SCV showed a higher level of intracellular persistence in endothelial cells than did the wild type (19).

The seven pinpoint isolates had a mutation in either ferrochelatase (*hemH*) or glutamyl tRNA reductase (*hemA*). Ferrochelatase is an enzyme that is localized in the cytoplasmic membrane in prokaryotes, where it catalyzes the terminal step in the biosynthesis of heme (23), whereas glutamyl tRNA reductase (*hemA*) participates in heme metabolism (24). It seems likely that this mutation causes the pinpoint phenotype, since addition of hemin causes reversion to the normal-size phenotype. Also, a deletion of *perR* (a regulon that mediates protection against reactive oxygen

species), which regulates several genes, including *hemA* in *L. monocytogenes*, resulted in the small-colony phenotype (12). It seems likely that a mutation in heme biosynthesis can cause the gentamicin resistance in the pinpoint isolates, as a similar generated mutant of *S. aureus* (*hemB*) showed typical SCV characteristics, including pinpoint growth and resistance to aminoglycosides (19). Interestingly, none of *S. aureus* SCVs isolated after exposure to triclosan were identified as hemin, menaquinone, or thymidine auxotrophs, and hence, the reason for their SCV phenotype is undetermined (10, 11). How triclosan targets the heme genes is not clear. However, others have demonstrated that exposure of *E. coli* and *P. aeruginosa* to inhibitory and subinhibitory concentrations of triclosan, respectively, affected the transcriptional response of operons involved in iron homeostasis (25, 26).

The *L. monocytogenes* pinpoint isolates had a very stable phenotype, as they did not change their morphology after growth on antibiotic-free medium. This is in contrast to clinical SCVs isolates of *S. aureus* which exhibit a high rate of reversion to the large-colony form when gentamicin is not present (6, 22, 27). Stable *S. aureus* SCVs can be generated by site-directed mutagenesis in *hemB* (19). Hence, the mutations in genes involved in hemin biosynthesis could cause the stability of the phenotype in *L. monocytogenes* pinpoint isolates.

The reason for the observed aminoglycoside resistance in the normal-size isolates is less clear, as all isolates had several mutations. However, several normal-size isolates had a mutation in ATP synthase F_1 subunit genes and one isolate in an ABC transporter. The F_0F_1 -ATPase is a multisubunit enzyme that synthesizes ATP aerobically and is important in the active transport of aminoglycosides across the cell membrane (28). *cydCD* encodes an ABC transporter that is involved in cytochrome assembly in *E. coli* (29) and hence in ATP generation. It seems likely that the resistance to the aminoglycosides in these isolates might be caused by a reduced uptake of the antibiotic. Interestingly, the two isolates with mixed populations only had one mutation, namely, in the geranyltranstransferase. This enzyme participates in the biosynthesis of isoprenoids and steroids which are part of the cell membrane in bacteria (reviewed in reference 30). It is not clear how this mutation can cause resistance to aminoglycosides, but it may be by reduced binding of the antibiotic, which is the first step in the active uptake of the aminoglycosides (28), or by reduced uptake. No obvious pattern between type of mutation and changed carbohydrate utilization could be found.

Triclosan, unlike other biocides, has a specific target when used at sublethal concentrations, namely, the enoyl-acyl carrier protein reductase, FabI (31). However, Seaman et al. (11) did not detect any mutations in *fabI* in triclosan-resistant *S. aureus* SCVs. Additional types of triclosan-mediated bactericidal activity, such as membrane integrity, interference with dehydrogenases/oxidoreductases, and interference with respiration, have been suggested (32, 33, 34, 35). Our sequencing data also suggest that sublethal concentrations of triclosan have multiple targets, since several genes were affected. We speculate that the interference with respiration that many of the detected mutations may have causes the aminoglycoside resistance due to a decreased uptake of the antibiotics. Similarly, several studies of *S. aureus* SCVs point to defects in pathways that are involved in electron transport (reviewed in reference 4).

To our knowledge, this is the first study to characterize a small-colony variant in *L. monocytogenes* that can be induced by sub-

lethal concentrations of triclosan. The *L. monocytogenes* pinpoint isolates were characterized by resistance to aminoglycosides, impaired growth under aerated conditions, increased growth during antibiotic stress, decreased hemolytic activity, and changed spectrum of carbohydrate utilization. The pinpoint phenotype seems to be caused by mutations in heme genes. Our study emphasizes that sublethal concentrations of triclosan likely have more targets than just *fabI* and that the biocide can cause resistance to antibiotics that are taken up by active transport. Further studies are needed to elucidate if *L. monocytogenes* pinpoint isolates could have any clinical impact, e.g., in persistent infections as seen for *S. aureus* SCVs (5, 6, 7, 19).

ACKNOWLEDGMENTS

We thank Jette Melchiorson for excellent technical assistance and Gitte M. Knudsen for good discussions.

This work was funded by the Program Committee for Food, Health and Welfare under the Danish Council for Strategic Research (project 2101-08-0030, Biocide Resistance, an Emerging Threat to Public Health) and by Danish Council for Independent Research, Technology and Production Sciences (FTP) grant 09-066098 (274-08-0531).

REFERENCES

- Mohan K, Gordon RC, Beaman TC, Belding RC, Luecke D, Edmiston C, Gerhardt P. 1977. Synergism of penicillin and gentamicin against *Listeria monocytogenes* in ex vivo hemodialysis culture. *J. Infect. Dis.* 135: 51–54. <http://dx.doi.org/10.1093/infdis/135.1.51>.
- Scheld WM, Fletcher DD, Fink FN, Sande MA. 1979. Response to therapy in an experimental rabbit model of meningitis due to *Listeria monocytogenes*. *J. Infect. Dis.* 140:287–294. <http://dx.doi.org/10.1093/infdis/140.3.287>.
- Christensen E, Gram L, Kastbjerg VG. 2011. Sublethal triclosan exposure decreases susceptibility to gentamicin and other aminoglycosides in *Listeria monocytogenes*. *Antimicrob. Agents Chemother.* 55:4064–4071. <http://dx.doi.org/10.1128/AAC.00460-11>.
- Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P, Herrmann M, Peters G. 2006. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat. Rev. Microbiol.* 4:295–305. <http://dx.doi.org/10.1038/nrmicro1384>.
- Kahl B, Herrmann M, Everding AS, Koch HG, Becker K, Harms E, Proctor R, Peters G. 1998. Persistent infection with small colony variant strains of *Staphylococcus aureus* in patients with cystic fibrosis. *J. Infect. Dis.* 177:1023–1029. <http://dx.doi.org/10.1086/515238>.
- Proctor RA, van Langevelde P, Kristjansson M, Maslow JN, Arbeit RD. 1995. Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. *Clin. Infect. Dis.* 20:95–102. <http://dx.doi.org/10.1093/clinids/20.1.95>.
- von Eiff C, Bettin D, Proctor RA, Rolaufts B, Lindner N, Winkelmann W, Peters G. 1997. Recovery of small colony variants of *Staphylococcus aureus* following gentamicin bead placement for osteomyelitis. *Clin. Infect. Dis.* 25:1250–1251. <http://dx.doi.org/10.1086/516962>.
- McNamara PJ, Proctor RA. 2000. *Staphylococcus aureus* small colony variants, electron transport and persistent infections. *Int. J. Antimicrob. Agents* 14:117–122. [http://dx.doi.org/10.1016/S0924-8579\(99\)00170-3](http://dx.doi.org/10.1016/S0924-8579(99)00170-3).
- Balwit JM, van Langevelde P, Vann JM, Proctor RA. 1994. Gentamicin-resistant, menadione and hemin auxotrophic *Staphylococcus aureus* persist within cultured endothelial cells. *J. Infect. Dis.* 170:1033–1037. <http://dx.doi.org/10.1093/infdis/170.4.1033>.
- Latimer J, Forbes S, McBrain AJ. 2012. Attenuated virulence and biofilm formation in *Staphylococcus aureus* following sublethal exposure to triclosan. *Antimicrob. Agents Chemother.* 56:3092–3100. <http://dx.doi.org/10.1128/AAC.05904-11>.
- Seaman PF, Ochs D, Day MJ. 2007. Small-colony variants: a novel mechanism for triclosan resistance in methicillin-resistant *Staphylococcus aureus*. *J. Antimicrob. Chemother.* 59:43–50. <http://dx.doi.org/10.1093/jac/dkl450>.
- Rea R, Hill C, Gahan CGM. 2005. *Listeria monocytogenes* PerR mutants display a small-colony phenotype, increased sensitivity to hydrogen peroxide, and significantly reduced murine virulence. *Appl. Environ. Microbiol.* 71:8314–8322. <http://dx.doi.org/10.1128/AEM.71.12.8314-8322.2005>.

13. Wulff G, Gram L, Ahrens P, Vogel BF. 2006. One group of genetically similar *Listeria monocytogenes* strains frequently dominate and persist in several fish slaughter- and smokehouses. *Appl. Environ. Microbiol.* 72: 4313–4322. <http://dx.doi.org/10.1128/AEM.02288-05>.
14. Dominguez Rodriguez LD, Vazquez Boland JA, Fernandez Garayzabal JF, Echalecu Tranchant P, Gomez-Lucia E, Rodriguez Ferri EF, Suarez Fernandez G. 1986. Microplate technique to determine hemolytic activity for routine typing of *Listeria* strains. *J. Clin. Microbiol.* 24:99–103.
15. Leimeister-Wächter M, Chakraborty T. 1989. Detection of listeriolysin, the thiol-dependent hemolysin in *Listeria monocytogenes*, *Listeria ivanovii*, and *Listeria seeligeri*. *Infect. Immun.* 57:2350–2357.
16. Holch A, Webb K, Lukjancenko O, Ussery D, Rosenthal BM, Gram L. 2013. Genome sequencing identifies two nearly unchanged strains of persistent *Listeria monocytogenes* isolated at two different fish processing plants sampled 6 years apart. *Appl. Environ. Microbiol.* 79:2944–2951. <http://dx.doi.org/10.1128/AEM.03715-12>.
17. Yegian D, Gallo G, Toll MW. 1959. Kanamycin resistant staphylococcus mutant requiring heme for growth. *J. Bacteriol.* 78:10–12.
18. Nielsen LN, Larsen MH, Skovgaard S, Kastbjerg VG, Westh H, Gram L, Ingmer H. 2013. *Staphylococcus aureus* but not *Listeria monocytogenes* adapt to triclosan and adaptation correlates with increased *fabI* expression and *agr* deficiency. *BMC Microbiol.* 13:177. <http://dx.doi.org/10.1186/1471-2180-13-177>.
19. von Eiff C, Heilmann C, Proctor RA, Woltz C, Peters G, Götz F. 1997. A site-directed *Staphylococcus aureus* *hemB* mutant is a small-colony variant which persists intracellularly. *J. Bacteriol.* 179:4706–4712.
20. Echave P, Tamarit J, Cabisco E, Ros J. 2003. Novel antioxidant role of alcohol dehydrogenase E from *Escherichia coli*. *J. Biol. Chem.* 278:30193–30198. <http://dx.doi.org/10.1074/jbc.M304351200>.
21. von Eiff C, McNamara P, Becker K, Bates D, Lei X-H, Ziman M, Bochner BR, Peters G, Proctor RA. 2006. Phenotype microarray profiling of *Staphylococcus aureus* *menD* and *hemB* mutants with the small-colony-variant phenotype. *J. Bacteriol.* 188:687–693. <http://dx.doi.org/10.1128/JB.188.2.687-693.2006>.
22. Sifri CD, Baresch-Bernal A, Calderwood SB, von Eiff C. 2006. Virulence of *Staphylococcus aureus* small colony variants in the *Caenorhabditis elegans* infection model. *Infect. Immun.* 74:1091–1096. <http://dx.doi.org/10.1128/IAI.74.2.1091-1096.2006>.
23. Ferreira GC, Franco R, Lloyd SG, Moura I, Moura JJG, Huynh BH. 1995. Structure and function of ferrochelatase. *J. Bioenerg. Biomembr.* 27:221–229. <http://dx.doi.org/10.1007/BF02110037>.
24. Freist W, Gauss DH, Söll D, Lapointe J. 1997. Glutamyl-tRNA synthetase. *Biol. Chem.* 378:1313–1329.
25. Chuanchuen R, Schweizer H. 2012. Global transcriptional responses to triclosan exposure in *Pseudomonas aeruginosa*. *Int. J. Antimicrob. Agents* 40:114–122. <http://dx.doi.org/10.1016/j.ijantimicag.2012.04.008>.
26. Bailey AM, Constantinidou C, Ivens A, Garvey MI, Webber MA, Colham N, Hobman JL, Wain J, Woodward MJ, Piddock LJV. 2009. Exposure of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium to triclosan induces a species-specific response, including drug detoxification. *J. Antimicrob. Chemother.* 64:973–985. <http://dx.doi.org/10.1093/jac/dkp320>.
27. Massey RC, Buckling A, Peacock J. 2001. Phenotypic switching of antibiotic resistance circumvents permanent costs in *Staphylococcus aureus*. *Curr. Biol.* 11:1810–1814. [http://dx.doi.org/10.1016/S0960-9822\(01\)00507-3](http://dx.doi.org/10.1016/S0960-9822(01)00507-3).
28. Taber HW, Mueller JP, Miller PF, Arrow AS. 1987. Bacterial uptake of aminoglycoside antibiotics. *Microbiol. Rev.* 51:439–457.
29. Poole RK, Gibson F, Wu G. 1994. The *cydD* gene product, component of a heterodimeric ABC transporter, is required for assembly of periplasmic cytochrome c and of cytochrome bd in *Escherichia coli*. *FEMS Microbiol. Lett.* 117:217–224. <http://dx.doi.org/10.1111/j.1574-6968.1994.tb06768.x>.
30. Ogura K, Koyama T. 1998. Enzymatic aspects of isoprenoid chain elongation. *Chem. Rev.* 98:1263–1278. <http://dx.doi.org/10.1021/cr9600464>.
31. McMurtry LM, Oethinger M, Levy SB. 1998. Triclosan targets lipid synthesis. *Nature* 394:531–532. <http://dx.doi.org/10.1038/28970>.
32. Boshoff HI, Myers TG, Copp BR, McNeil MR, Wilson MA, Barry CE. 2004. The transcriptional responses of *Mycobacterium tuberculosis* to inhibitors of metabolism. *J. Biol. Chem.* 279:40174–40184. <http://dx.doi.org/10.1074/jbc.M406796200>.
33. Gomez Escalada M, Russell AD, Maillard J-Y, Ochs D. 2005. Triclosan-bacteria interactions: single or multiple target sites? *Lett. Appl. Microbiol.* 41:476–481. <http://dx.doi.org/10.1111/j.1472-765X.2005.01790.x>.
34. Villalain J, Mateo CR, Aranda FJ, Shapiro S, Micol V. 2001. Membranotropic effects of the antibacterial agent triclosan. *Arch. Biochem. Biophys.* 390:128–136. <http://dx.doi.org/10.1006/abbi.2001.2356>.
35. Yu BJ, Kim JA, Ju HM, Choi S-K, Hwang SJ, Park S, Kim E, Pan J-G. 2012. Genome-wide enrichment screening reveals multiple targets and resistance genes for triclosan in *Escherichia coli*. *J. Microbiol.* 50:785–791. <http://dx.doi.org/10.1007/s12275-012-2439-0>.