

# Genetic Characterization of Plasmid-Associated Triphenylmethane Reductase in *Listeria monocytogenes*

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The enzyme triphenylmethane reductase (TMR) reduces toxic triphenylmethane dyes into colorless, nontoxic derivatives, and TMR-producing microorganisms have been proposed as bioremediation tools. Analysis of the genome of *Listeria monocytogenes* H7858 (1998-1999 hot dog outbreak) revealed that the plasmid (pLM80) of this strain harboring a gene cassette (*bcrABC*) conferring resistance to benzalkonium chloride (BC) and other quaternary ammonium disinfectants also harbored a gene (*tmr*) highly homologous to TMR-encoding genes from diverse Gram-negative bacteria. The pLM80-associated *tmr* was located two genes downstream of *bcrABC* as part of a putative IS1216 composite transposon. To confirm the role of *tmr* in triphenylmethane dye detoxification, we introduced various *tmr*-harboring fragments of pLM80 in a pLM80-cured derivative of strain H7550, from the same outbreak as H7858, and assessed the resistance of the constructs to the triphenylmethane dyes crystal violet (CV) and malachite green. Transcriptional and subcloning data suggest that the regulation of TMR is complex. Constructs harboring fragments spanning *bcrABC* and *tmr* were CV resistant, and in such constructs *tmr* transcription was induced by sublethal levels of either BC or CV. However, constructs harboring only *tmr* and its upstream intergenic region could also confer resistance to CV, albeit at lower levels. Screening a panel of BC-resistant *L. monocytogenes* strains revealed that all those harboring *bcrABC* and adjacent pLM80 sequences, including *tmr*, were resistant to CV and decolorized this dye. The findings suggest a potential role of TMR as a previously unknown adaptive attribute for environmental persistence of *L. monocytogenes*.

*Listeria monocytogenes* is a food-borne facultative intracellular pathogen that is able to contaminate a variety of ready-to-eat food products and food processing facilities (1-4). It has been hypothesized that biofilm formation, resistance to disinfectants, resistance to *Listeria*-specific viruses, and the ability to replicate at low temperatures are among the major attributes that contribute to the prevalence and persistence of *L. monocytogenes* in food-related environments (5-10). The current list of adaptive attributes of *L. monocytogenes* is not exhaustive. Substantial effort has been directed toward the identification of new adaptations and niches that might lead to persistence of *L. monocytogenes* in the environment.

Triphenylmethane dyes such as crystal violet (CV) and malachite green have had numerous industrial applications, e.g., as dyes in the textile industry or antifungal agents in aquaculture. Such uses have been accompanied with release into the environment as industrial effluents (11, 12). Due to increasing interest in bioremediation tools for these dyes, a number of studies have utilized the ability of microbes, including fungi and bacteria, to enzymatically decolorize and detoxify the dyes (13). Triphenylmethane reductase (TMR) is one such enzyme produced by diverse Gram-negative bacteria such as *Citrobacter* (accession number AY756172), *Aeromonas* (accession number EF010984), and *Pseudomonas* (accession number FJ649187) spp. (14-17). A TMR produced by *Citrobacter* sp. strain MY-5 (accession number AY756172) and by *Pseudomonas* sp. has been characterized in a heterologous host (*Escherichia coli*) (14, 16-19); however, limited information is currently available on the ecological importance and role of TMR in its native hosts or on genes encoding similar TMRs in other bacteria.

Genome analysis of *L. monocytogenes* strain H7858 from the 1998-1999 listeriosis outbreak involving contaminated hot dogs revealed a large (ca. 80 kb) plasmid, pLM80, which harbored a

gene cassette (*bcrABC*) conferring resistance to benzalkonium chloride (BC) and other quaternary ammonium disinfectants (20-22). Analysis of *bcrABC* from strain H7550, from the same 1998-1999 outbreak as strain H7858, revealed that *bcrABC* (1.2 kb) was transcribed from a canonical promoter and was part of a larger, 3.8-kb transcript. Interestingly, the last gene in this operon, two genes downstream from *bcrC*, was annotated as a putative TMR (21, 22). However, functional confirmation of the involvement of this gene (*tmr*) in dye detoxification in *L. monocytogenes* has been lacking. In the present study, we characterized *tmr* in *L. monocytogenes* H7550 and assessed *tmr* prevalence and dye detoxification potential among *L. monocytogenes* isolates.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The *L. monocytogenes* strains used for subcloning of *tmr*-harboring fragments of pLM80 are listed in Table 1 and were grown in brain heart infusion broth (BHI; Becton Dickinson) or on BHI agar (BHI with 1.2% Bacto-agar [Becton Dickinson]). Other *L. monocytogenes* strains used in the present study were from our laboratory's *Listeria* strain collection at North Carolina State University. These included 18 strains (14 environmental and 4 from human listeriosis) of serotype 1/2a or 3a, 12 environmental strains of serotype 1/2b or 3b, 3 environmental strains of serotype 1/2c or 3c, and 14 strains (2 environmental and 12 from human listeriosis) of serotype 4b. Environmental strains were from turkey processing plants (8). *Escherichia coli* strains were

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TABLE 1 *L. monocytogenes* strains used in this study

<i>L. monocytogenes</i> strain <sup>a</sup>	CV MIC <sup>b</sup> (μg/ml)	Source or reference
H7550	20	22
H7550-Cd <sup>S</sup>	2.5	22
Cd <sup>S</sup> -pPL81	15	This study
Cd <sup>S</sup> -pPL82	10	This study
Cd <sup>S</sup> -pPL83	17.5	This study
Cd <sup>S</sup> -pPL84	10	This study
Cd <sup>S</sup> -pPL2	2.5	This study
Cd <sup>S</sup> -pPLBC	2.5	This study

<sup>a</sup> Strains harboring various integrated constructs are described in Materials and Methods.

<sup>b</sup> MICs were determined in two independent trials as described in Materials and Methods. Identical values were obtained from the two trials for all strains with the exception of Cd<sup>S</sup>-pPL83 (MIC = 20 and 15 μg/ml in trials 1 and 2, respectively).

grown at 30°C in Luria-Bertani (LB) broth (Becton Dickinson) or on LB broth supplemented with 1.2% Bacto agar. When indicated, the antibiotics used for *Listeria* were nalidixic acid (20 μg/ml) and chloramphenicol (6 μg/ml), while chloramphenicol (25 μg/ml) was used for *E. coli*. The antibiotics were purchased from Sigma-Aldrich (St. Louis, MO).

**BC and crystal violet (CV) resistance and MIC determinations.** BC resistance was determined as described before (8). For CV susceptibility determination, a single colony from a blood agar plate (Remel) was inoculated in 100 μl of BHI broth, and 5 μl was spotted in duplicate onto BHI agar supplemented with 15 μg/ml CV (Fisher, Fair Lawn, NJ). The plates were incubated at 37°C for 48 h. Strains H7550 and its pLM80-cured derivative H7550-Cd<sup>S</sup> (22) were used as resistant and susceptible controls, respectively. For CV MIC determinations, overnight cultures were grown at 37°C (ca. 10<sup>9</sup> CFU/ml), and 3 μl was spotted in duplicate onto the surfaces of BHI agar plates supplemented with variable concentrations of CV (0, 2.5, 5, 10, 15, and 20 μg/ml). The plates were incubated at 37°C for 48 h, and the MIC was defined as the lowest assessed concentration of CV that prevented confluent growth on the spots.

**PCR and recombinant plasmid constructs.** The locations of the primers used to construct the recombinant plasmids are indicated in Fig. 1. PCR analyses employed the TaKaRa ExTaq kit (TaKaRa, Madison, WI) and a T1 thermal cycler (Biometra, Gottingen, Germany). Primers p1/p2 and 0067F/0067R were used for *bcrABC* and for *tmr*, respectively (22, 23).

For recombinant constructs, the primers B1 (5'-GACTCCCGGGGA TTCTGGAAACATCCCTATC-3') and 66R (5'-GGTTCGCGGGGGCAAT GCAATTGTCTATATCAAC-3'; the XmaI sites are underlined) were used to amplify a 4,158-bp fragment extending from 105 nucleotides (nt) upstream of *bcrA*, along with the canonical promoter (P1), to 281 nt downstream of the *tmr* stop codon. This fragment was digested with XmaI (New England BioLabs, Beverly, MA) and ligated with T4 DNA ligase (Promega, Madison, WI) into similarly digested pPL2 (24) to produce pPL81. The primers TMR\_F (5'-GACTGGTACCCAATCAATTACGCA TTTGTACTTG-3') and 66R2 (5'-GACTGGTACCGGCAATGCAATTG TCTATATCAAC-3'; the KpnI sites are underlined) were used to amplify a 1,571-bp fragment harboring the entire intergenic space (426 bp) upstream of the start codon of *tmr* (including P2, located 175 nt upstream of the *tmr* start codon), along with *tmr* and 281 nt downstream of the *tmr* stop codon. This fragment was restricted with KpnI (New England BioLabs) and then ligated into similarly digested pPL2, resulting in pPL82 (Fig. 1). The primers BcF3 (5'-GACTGGTACC TTCAATTAGATCGAG GCACG-3'; the KpnI site is underlined) and 66R2 were used to amplify a 4,953-bp fragment that harbored the entire (850 nt) intergenic sequence upstream of *bcrA* and 257 nt downstream of the *tmr* intergenic region. This fragment was restricted with KpnI and was ligated into similarly digested pPL2, resulting in pPL83 (Fig. 1). The primers TMR2\_F (5'-GA CTGGTACC TCCAGTCTTCAATTGCGGCC-3'; the KpnI site is underlined) and 66R2 were used to amplify a 1,358-bp fragment harboring *tmr*, together with its upstream (213 bp, including P2) and downstream (281 bp) intergenic regions. The fragment was restricted with KpnI (New England BioLabs) and ligated into similarly digested pPL2, resulting in pPL84. The primers TMR3\_F (5'-GACTGGTACCGGAGGCATTACCTTGTT TAG-3'; the KpnI site is underlined) and 66R2 were used to amplify a fragment (1,245 bp) with a shorter (100-bp) intergenic region upstream of *tmr*, thus lacking P2. This fragment was restricted with KpnI (New

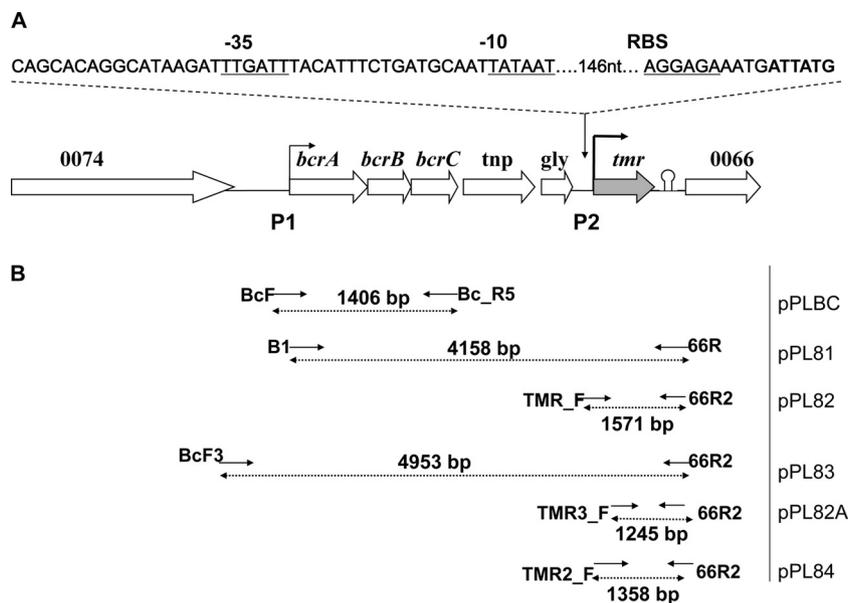


FIG 1 Genetic characterization of *tmr* in *L. monocytogenes*. (A) Schematic diagram of the *tmr*-harboring region of pLM80 (20). P1 and P2 represent the two putative promoters where P1 is a canonical promoter upstream of *bcrABC* described before (22), and P2 is immediately upstream of *tmr*. (B) Recombinant plasmids harboring different *tmr* segments. Solid arrows indicate the position and orientation of the primers used for recombinant plasmid construction. The plasmids were introduced in the pLM80-cured derivative H7550-Cd<sup>S</sup> and integrated into the chromosome, as described in Materials and Methods.

England BioLabs) and ligated into similarly digested pPL2, resulting in pPL82A (Fig. 1). Recombinant plasmids were electroporated first into *E. coli* DH5 $\alpha$ , followed by selection on LB medium supplemented with 15  $\mu$ g/ml CV for 24 to 48 h at 30°C, until the medium was decolorized. The primers BcF and BC\_R5 (22) were used to amplify a 1,406-bp fragment harboring the 1,231-bp *bcrABC*, along with 105 bp upstream of *bcrA* and 70 bp downstream of *bcrC*. This fragment was digested with SacI/XmaI (restriction sites harbored on the primers) and ligated into similarly digested pPL2, resulting in pPLBC (Fig. 1). Like the other plasmids, pPLBC was first electroporated in *E. coli* DH5 $\alpha$ , followed by selection on LB agar supplemented with chloramphenicol at 25  $\mu$ g/ml (24). Plasmids from the *E. coli* DH5 $\alpha$  transformants were confirmed with PCR and restriction analysis, followed by electroporation into *E. coli* S17-1. The recombinant plasmids pPL81, pPL82, pPL83, pPL84, and pPLBC and the empty vector (pPL2) were then mobilized into H7550-Cd<sup>S</sup> via conjugation and integrated into the chromosome, as described previously (22, 24). Transconjugants were selected on BHI agar plates supplemented with chloramphenicol (6  $\mu$ g/ml) and nalidixic acid (20  $\mu$ g/ml) at 30°C for 2 to 3 days and confirmed using PCR as described previously (24).

**Decolorization curves.** Overnight cultures (37°C in BHI) of *L. monocytogenes* strains H7550, H7550-Cd<sup>S</sup> harboring pPL2 (Cd<sup>S</sup>-pPL2), and derivatives of H7550-Cd<sup>S</sup>, i.e., pPL81, pPL82, pPL83, and pPL84 were diluted 1:20 in BHI supplemented with 15  $\mu$ g/ml CV in separate wells of 24-well plates (Corning, NY). The inoculated plates were incubated at 37°C and the decrease in  $A_{590}$  of the cultures was measured at several intervals using a spectrophotometer (SmartSpec 3000; Bio-Rad, Hercules, CA) until the CV was completely decolorized. Experiments were done in at least two independent trials. For growth assessments, overnight cultures were diluted 1:20 in BHI, followed by incubation at 37°C. The  $A_{600}$  was recorded using a spectrophotometer (SmartSpec 3000) at hourly intervals for up to 8 h. To assess the impact of CV on growth of H7550 and Cd<sup>S</sup>-pPL2, cultures (1:20 dilutions in BHI, as described above) were incubated at 37°C in the presence of CV (7.5  $\mu$ g/ml), and CFU were determined by plating dilutions at 4.5, 24 and 48 h. The assay was done in two independent trials.

**Transcriptional analyses.** Bacteria were grown in BHI at 37°C until mid-stationary phase ( $A_{600} \sim 0.7$  to 0.9, measured using a spectrophotometer [SmartSpec 3000]). The cultures were then divided into two equal portions, one of which was supplemented with 15  $\mu$ g/ml CV, while the other was left untreated and incubated at 37°C for 30 min. The total RNA was isolated from the CV-treated and untreated cultures as described previously (22, 23, 25). RNA was isolated in three independent trials and reverse transcribed as described before (22, 23, 25). The gene-specific cDNA was reverse transcribed using 0067-R for *tmr* (23) and s2 for the housekeeping gene *spoVG*, which was used as a reference (22, 25, 26).

Quantitative real-time PCR (qPCR) was used to assess the expression of *tmr* in the presence or absence of CV using the Applied Biosystems 7500 Fast real-time PCR system according to the manufacturer's instructions (Applied Biosystems, Foster City, CA) and as described before (25). The primers qTMR\_F (5'-GAGTCTGGAGCAATTGTTAC-3'), qTMR\_R (5'-CAAGTTATACGTTTTGTTTCATG-3'), and TaqMan probe qTMR\_P (5'-FAM-TTCCTCTGTAAGAACCGTTGCG3'-BHQ1) were used for *tmr*; the primers and probe used for *spoVG* were as described before (25). Assays were performed in triplicate and in at least three independent trials.

**Statistics.** Expression levels for *tmr* were reported relative to those of the housekeeping gene *spoVG*, used as an internal control (26). The quantification of *tmr* transcript levels for each strain and treatment (with or without CV) was done by normalizing the *tmr*  $C_T$  values to those of *spoVG* for the same strain and treatment. The resulting values ( $\Delta C_T$ ) were used ( $2^{-\Delta C_T}$ ) to express the fold change between *tmr* and *spoVG* transcript levels, assuming a gene amplification efficiency for both *tmr* and *spoVG* of 2 (25, 27).

A mixed linear model (SAS, Cary, NC) was fit to the  $\Delta C_T$  measure-

ments in order to test hypotheses regarding factorial effects of strain and CV treatment. In particular, random effects were included for trial and trial-by-treatment interaction so that appropriate error terms were used in tests for these hypotheses. Residual diagnostics did not reveal any violation of the assumptions of normally distributed  $\Delta C_T$  measurements and homogeneity of variance necessary for statistical inference. To test for equality of gene expression across strains separately with or without the CV treatment, a nested model was fit in which the strain effect was nested within the CV factor.

## RESULTS AND DISCUSSION

**Distribution of *tmr* suggests recent transfer from Gram-negative bacteria to *Listeria*, possibly in triphenylmethane dye-contaminated environments.** In *L. monocytogenes* H7858 the putative *tmr* (864 bp) was harbored on the large plasmid pLM80 (LMOh7858\_plm80\_0067, accession number NZ\_AADR000000000), two genes downstream of the BC resistance cassette *bcrABC* (20–22; Fig. 1). The gene had unusually high GC content (45%) in comparison to the genome average (38%) of *L. monocytogenes*. BLAST analysis of other *Listeria* genomes revealed conserved (100% identity) *tmr* sequences in a pLM80-like (~80-kb) plasmid harbored by *L. monocytogenes* J0161 (serotype 1/2a, implicated in a 2000 outbreak involving turkey deli meats) (21) and a ~149-kb plasmid from strain *L. monocytogenes* N1-011A; sequences with 100% identity were also detected in *L. monocytogenes* 11GZL18 (serotype 1/2c strain from raw pork), *L. monocytogenes* Lm1986, and *L. seeligeri* N1-067, isolated from trout brine (28). However, various homologs with 99 to 100% nucleotide identity over the entire length of the gene were also detected among high-GC-content Gram-negative bacteria from wastewater treatment systems, aquaculture water, and soil in a textile effluent treatment facility. These included *Citrobacter* sp. strain MY-5 [66% GC], accession number AY756172; *Pseudomonas* sp. [63% GC], accession number FJ649187; *Aeromonas hydrophila* subsp. *decolorationis* [61% GC], accession number EF010984; *Comamonas* sp. [52% GC], accession number JN648092; and *Delftia* sp. [66% GC], accession number NC\_019312. With the exception of *Listeria*, *tmr* homologs were under-represented in Gram-positive bacteria, being reported only in *Geobacillus* sp. (43% GC; 98 to 99% identity over 86% of the gene).

Analysis of the intergenic sequence upstream of *tmr* in pLM80 revealed a putative promoter (P2: –10 TATAAT; –35 TTGATT) 146 nt upstream of the *tmr* start codon (Fig. 1). The upstream 395-nt intergenic region, including P2, was conserved in several of the Gram-negative *tmr* homologs. The promoter sequence for P2 has not been characterized before since the Gram-negative *tmr* homologs were expressed in a heterologous host under a non-native promoter (14, 16, 17). Analysis of the 239-nt intergenic sequence downstream of *tmr* in pLM80 also revealed that this sequence was conserved (100% identity) in the Gram-negative *tmr* homologs, including the previously identified rho-independent terminator 110 nt downstream of the *tmr* stop codon (22). In certain cases (*Comamonas* sp. strain KV11 and KV36, *Delftia* sp. strain KV29 and uncultured bacterium pGNB1) the downstream conserved sequences extended beyond the 239-nt intergenic region downstream of *tmr* to include a hypothetical gene, LMOh7858\_plm80\_0066 (34% GC) (Fig. 1).

The high-level conservation of *tmr* and its upstream and downstream sequences between *L. monocytogenes* and the Gram-negative homologs, together with the unusually high GC content of *tmr* and its over-representation among Gram-negative bacteria,

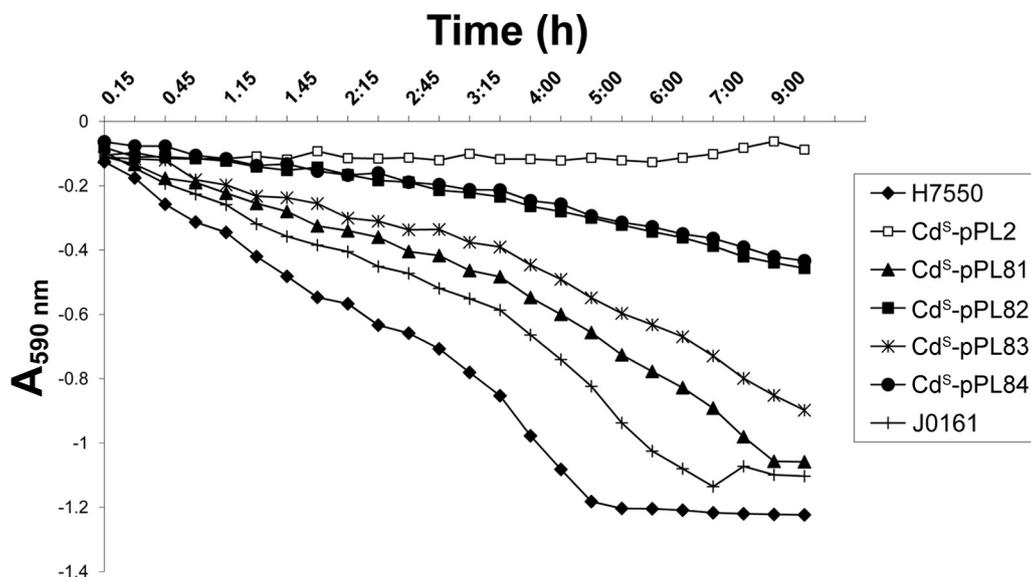


FIG 2 *L. monocytogenes* strain H7550 and the derivatives of H7550-Cd<sup>S</sup> harboring different *tmr* segments decolorize CV (15 µg/ml) at different rates. The experimental details are provided in Materials and Methods. This graph is a representative experiment from three independent trials.

suggest recent transfer of these sequences from Gram-negative bacteria to *Listeria*. The Gram-negative bacteria themselves likely acquired *tmr* from other sources, since their genome GC content (52 to 66%, as discussed above) was significantly higher than that for *tmr* (45%) (14, 16). It is tempting to speculate that transfer of *tmr* took place and conferred selective advantages to *Listeria* in environments contaminated with triphenylmethane dyes, such as those associated with aquaculture, wastewater treatment, and textile industry effluents.

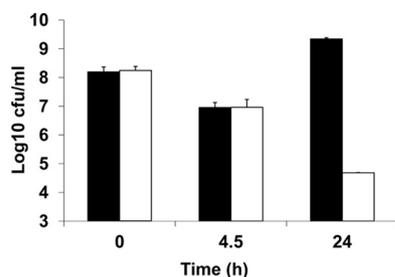
***tmr* confers CV decolorization and CV resistance to *L. monocytogenes* strain H7550.** Preliminary evidence for the role of *tmr* in CV resistance and decolorization was obtained from comparisons between strain H7550 (harboring *tmr* on pLM80) and its pLM80-cured derivative, H7550-Cd<sup>S</sup>. After overnight incubation at 37°C on BHI agar with 15 µg/ml CV (CV-15), confluent growth with a zone of decolorized CV was seen for H7550, while no growth or decolorization could be noted for H7550-Cd<sup>S</sup> (data not shown). The CV MICs for H7550 and H7550-Cd<sup>S</sup> were 20 and 2.5 µg/ml, respectively. These strains were also tested with a different triphenylmethane dye, malachite green. Strain H7550 grew noticeably better than H7550-Cd<sup>S</sup> on agar with this dye (data not shown). Differences were more pronounced and consistent with CV, and the latter was used for the remainder of the study.

Cloning of *tmr* with different upstream and downstream sequences resulted in the chromosomally integrated plasmids shown in Fig. 1. Strain Cd<sup>S</sup>-pPL2 (H7550-Cd<sup>S</sup> harboring the integrated empty vector, pPL2) was susceptible to CV (MIC = 2.5 µg/ml) and unable to decolorize CV-15 (Table 1 and Fig. 2). However, the genetically complemented strains Cd<sup>S</sup>-pPL81 and Cd<sup>S</sup>-pPL83, harboring *tmr* and upstream genes (Fig. 1), recovered the ability to grow on CV (MIC ≥ 15 µg/ml) and to decolorize the dye (Table 1 and Fig. 2). Resistance to CV was also restored (MIC = 10 µg/ml; Table 1) in the complemented strains that harbored only *tmr*, together with either the entire (426 nt) or a portion (213 nt) of the upstream intergenic sequence (Cd<sup>S</sup>-pPL82 and Cd<sup>S</sup>-pPL84, respectively) (Fig. 1). However, pPL82A harboring only *tmr* with

100 nt upstream of *tmr* start codon and thus lacking P2 failed to yield *E. coli* DH5α recombinants on CV-15. These data suggest that *tmr* can be expressed from the P1 promoter, together with *bcrABC* and other genes in the *bcrABC* multicistronic unit, as previously reported (22). Further studies are needed to determine whether *tmr* in pLM80 and constructs such as pPL81 and pPL83 (which harbor both P1 and P2) is expressed from P1 only or from both P1 and P2. Furthermore, it is currently not known whether certain environmental signals or conditions impact the relative contribution of P1 and P2 in pLM80 and constructs harboring both P1 and P2. However, CV resistance and decolorization conferred by pPL82 and pPL84 (which harbor only P2) suggest that *tmr* can also be expressed from its own promoter, P2.

Among the complemented integrated derivatives, Cd<sup>S</sup>-pPL81 exhibited a consistently higher CV decolorization efficiency than Cd<sup>S</sup>-pPL83 (Fig. 2). This was surprising, since Cd<sup>S</sup>-pPL81 and Cd<sup>S</sup>-pPL83 had similar CV MICs (Table 1). Cd<sup>S</sup>-pPL83 harbored the entire (795-nt) intergenic region upstream of *bcrA*, whereas Cd<sup>S</sup>-pPL81 only included 105 nt upstream of *bcrA*; both constructs included the promoter P1 (Fig. 1). The higher decolorization efficiency of Cd<sup>S</sup>-pPL81 suggests the possibility of negative regulatory controls based on sequences within portions of the intergenic region present in Cd<sup>S</sup>-pPL83 but lacking in Cd<sup>S</sup>-pPL81. Indeed, this intergenic region is unusually long (795 nt), suggesting potential for regulatory involvement, e.g., via small noncoding RNAs (10). The decolorization potential of Cd<sup>S</sup>-pPL82 and Cd<sup>S</sup>-pPL84, although identical, was lowest among the complemented strains (Fig. 2). Together, these data suggest that, even though *tmr* can indeed be expressed on its own likely via P2 (as in pPL82 and pPL84), such expression is at lower levels than those observed with the multicistronic message that includes *bcrABC* and the other genes (as in pPL81 and pPL83).

Previous analysis of the *bcrABC* region indicated that *bcrABC* was transcribed together with several downstream genes, of which *tmr* was the last in the operon (22). It is tempting to speculate that the current location of *tmr* in the *bcrABC* region, and under con-



**FIG 3** Decolorization of crystal violet renders the dye nontoxic for *L. monocytogenes*. Black bars represent CFU of H7550 upon exposure to CV (15 µg/ml), whereas white bars represent CFU of Cd<sup>S</sup>-pPL2 upon exposure to CV (15 µg/ml). These data are based on two independent trials.

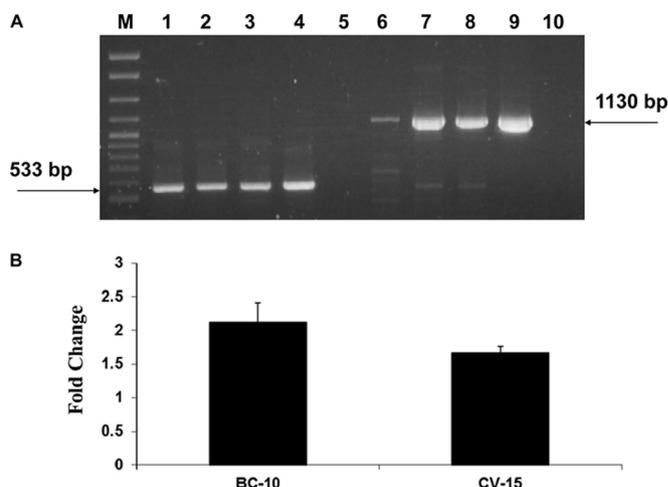
tol by P1, was selected upon due to the higher levels of expression observed in the presence of both P1 and P2.

Even though CV resistance and decolorization potential was restored in Cd<sup>S</sup>-pPL81, Cd<sup>S</sup>-pPL82, Cd<sup>S</sup>-pPL83, and Cd<sup>S</sup>-pPL84, both the MICs and the decolorization potential remained below those observed with the wild-type strain H7550 harboring pLM80 (Table 1 and Fig. 2). Copy number differences may account for this finding, since in the complemented strains the genes were integrated as single copies in the chromosome. It is also possible that pLM80 harbors additional, as-yet-uncharacterized determinants that contribute to CV resistance and decolorization.

As expected by the documented role of *bcrABC* in BC resistance, only Cd<sup>S</sup>-pPL81 and Cd<sup>S</sup>-pPL83 acquired BC resistance (BC 20 µg/ml), whereas Cd<sup>S</sup>-pPL82 and Cd<sup>S</sup>-pPL84 remained BC susceptible (data not shown). Strain Cd<sup>S</sup>-pPLBC harboring just P1 and *bcrABC* restored resistance to BC but not to CV and did not decolorize CV-15 or confer resistance to the dye (data not shown).

No differences were found in the growth rates of H7550, H7550-Cd<sup>S</sup>, and the H7550-Cd<sup>S</sup> complemented derivatives in BHI without CV (data not shown). After a 4.5-h exposure of *L. monocytogenes* H7550 and Cd<sup>S</sup>-pPL2 to 7.5 µg/ml CV, both strains exhibited a decrease in viability (~2-log<sub>10</sub> reduction in CFU/ml) (Fig. 3). During this period, *L. monocytogenes* H7550 completely decolorized CV and resumed growth, as evidenced by higher CFU at 24 h; in contrast, no decolorization was detected for Cd<sup>S</sup>-pPL2, and the survival of this strain continued to decline, with an ~4.5-log<sub>10</sub> reduction by 24 h (Fig. 2 and 3). Assessing the CFU/ml at 48 h indicated further reductions (~6-log<sub>10</sub> reduction) in Cd<sup>S</sup>-pPL2 but not in strain H7550 (data not shown). Together with the recovery of CV tolerance and decolorization potential by the genetically complemented strains, these findings indicate that *tmr*-mediated decolorization of CV results in detoxification of this dye for *L. monocytogenes*.

**The transcript levels of *bcrABC* and *tmr* increased in the presence of CV.** Previous data indicated that *tmr* was part of a 3.8-kb transcript that also included *bcrABC* (mediating resistance to BC). The amounts of this polycistronic transcript increased in the presence of sublethal amounts of BC (22, 23). To determine whether the exposure to CV could also result in enhanced amounts of this transcript, we used reverse transcription-PCR (RT-PCR) to assess the impact of CV treatment on the expression of *bcrABC*. The exposure of H7550 to CV-15 indeed resulted in enhanced amounts of the *bcrABC* transcript, even though these amounts were lower than observed by exposure to BC at 10 µg/ml



**FIG 4** Transcription of *bcrABC* is induced by CV (15 µg/ml). (A) RT-PCR of *spoVG* and *bcrABC*. Lanes 1 to 5, RT-PCR of *spoVG* using primer s2 (for cDNA) and primers s1 and s2 for PCR (22, 23) as follows: lane 1, H7550 in the absence of BC or CV; lane 2, H7550 exposed to BC-10 µg/ml; lane 3, H7550 exposed to CV (15 µg/ml); lanes 4 and 5, H7550 genomic DNA and total RNA, respectively, used as a positive and negative controls for RT-PCR. An arrow points to the expected *spoVG* PCR product of 533 bp. Lanes 6 to 10, RT-PCR of *bcrABC* using primer p2 (for cDNA) and primers p1 and p2 for PCR (22, 23), as follows: lane 6, H7550 in the absence of BC or CV; 7, H7550 exposed to BC (10 µg/ml); 8, H7550 exposed to CV (15 µg/ml); 9 and 10, H7550 genomic DNA and total RNA, respectively, used as a positive and negative control for RT-PCR. Arrow points to the expected *bcrABC* PCR product of 1130 bp. M, 100- to 2,686-bp DNA molecular marker XIV (Roche). The CV and BC exposures were for 30 min, as described in Materials and Methods. The product in lane 6 represents the baseline levels of *bcrABC*, as previously reported (22). (B) Fold change in transcript level of *bcrABC* upon exposure to BC (10 µg/ml) and CV (15 µg/ml). The fold change was calculated using the ImageJ software (rsb.info.nih.gov/ij) as described previously (22).

(Fig. 4) (22). These findings suggest that *bcrABC* expression could be induced not only by BC but also by CV.

qPCR analysis with H7550 revealed that *tmr* was expressed at low levels in the absence of CV, but expression increased significantly (2.6-fold;  $P = 0.012$ ) upon CV exposure. Increases were also noted for the integrated constructs Cd<sup>S</sup>-pPL81 (4.3-fold,  $P = 0.003$ ) and Cd<sup>S</sup>-pPL83 (3-fold,  $P = 0.005$ ) (Fig. 5). In spite of repeated efforts, we were unable to obtain RNA of sufficient quantity and quality from Cd<sup>S</sup>-pPL82 and Cd<sup>S</sup>-pPL84 upon exposure to CV-15, possibly due to increased cell death due to CV exposure even during the 30-min incubation. Thus, the impact of CV on expression of *tmr* under its own promoter could not be assessed. The baseline levels of *tmr* in the absence of CV did not differ significantly between H7550 and any of the complemented derivatives (data not shown).

**Presence of *tmr* is correlated with *bcrABC* and resistance to CV decolorization in *L. monocytogenes* strains of diverse serotypes.** PCR analysis of 47 strains of diverse serotypes and of environmental or clinical origins, as described in Materials and Methods (including 34 strains resistant to both CV and BC, 9 strains resistant only to BC, and 4 strains susceptible to both BC and CV), confirmed that all 34 CV-resistant strains harbored *tmr*, as well as *bcrABC*, whereas CV-susceptible strains were negative for *tmr*, regardless of whether they were BC resistant or not (data not shown). Analysis of numerous other *L. monocytogenes* strains has failed to identify any that are CV resistant without also being re-

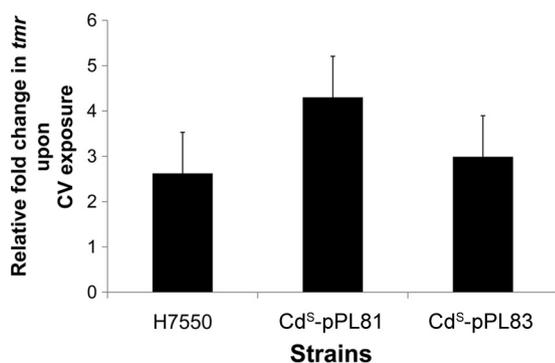


FIG 5 Relative increase in *tmr* transcript levels upon exposure to CV (15  $\mu\text{g/ml}$ ) in H7550 and H7550-Cd<sup>s</sup> derivatives. Quantification of *tmr* transcripts was performed using qRT-PCR as described in Materials and Methods. The *tmr* transcript levels were first normalized to those of *spoVG* from the same treatments. These normalized *tmr* transcript levels were used to calculate the relative fold increase in *tmr* upon exposure to CV (15  $\mu\text{g/ml}$ ). These data were computed from three independent trials.

sistant to BC (data not shown). Previous data have indeed shown that strains harboring conserved *bcrABC* and adjacent sequences, including *tmr*, had a pLM80-like *bcrABC* region and were of diverse serotypes and sources (23). The findings are in agreement with BLAST analysis of *L. monocytogenes* genomes discussed above, which revealed *tmr* only when *bcrABC* was also present. Additional analysis of recently sequenced *L. monocytogenes* genomes ( $n = 100$ ) from the Sequence Read Archive database (<http://www.ncbi.nlm.nih.gov/sra>) further supports these findings (data not shown).

In conclusion, our findings suggest that in *L. monocytogenes* CV resistance is associated with *tmr* and that this gene is typically encountered as a member of a multisubstrate resistance region on pLM80 and related plasmids. In pLM80 this region confers resistance to quaternary ammonium disinfectants, triphenylmethane dyes, and the heavy metal cadmium and appears to be a component of a composite transposon (20–22). Such genetic attributes associated with mobility likely account for the observed dissemination of *tmr* and the other resistance determinants among strains of diverse serotypes and sources.

It is intriguing that *tmr* appears to have been introduced into *Listeria* from a Gram-negative source and that it was incorporated into the resistance region in a way that allows its transcription not only from its own promoter, conserved in numerous triphenylmethane dye-resistant Gram-negative bacteria, but also utilizing the *bcrABC* promoter for expression. This genetic arrangement appears to have resulted in higher levels of *tmr* expression and was likely selected in environments with high triphenylmethane dye burdens. Findings from the current study will be useful in elucidating adaptations and evolution of *Listeria* in such ecosystems and in future assessments of the potential role of *tmr* in additional adaptations, including virulence.

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