JOURNAL OF BACTERIOLOGY, Jan. 1999, p. 666–669 Vol. 181, No. 2
0021-9193/99/$04.00+0

Targeted Mutagenesis by Duplication Insertion in the Radioresistant Bacterium *Deinococcus radiodurans*: Radiation Sensitivities of Catalase (*katA*) and Superoxide Dismutase (*sodA*) Mutants

LYE MENG MARKILLIE, SUSAN M. VARNUM, PRESTON HRADECKY, AND KWONG-KWOK WONG*

Pacific Northwest National Laboratory, Molecular Biosciences, Richland, Washington 99352

Received 21 August 1998/Accepted 27 October 1998

***Deinococcus radiodurans* R1 is extremely resistant to both oxidative stress and ionizing radiation. A simple and general targeted mutagenesis method was developed to generate catalase (*katA*) and superoxide dismutase (*sodA*) mutants. Both mutants were shown to be more sensitive to ionizing radiation than the wild type.**

Deinococcus (*Micrococcus*) *radiodurans* R1 has extreme resistance to genotoxic chemicals, oxidative damage, high levels of ionizing and UV radiation, and desiccation (4, 7, 12, 19). It has been suggested that the extreme resistance to ionizing radiation is attributable to an effective DNA repair system and a special chromosome structure (8, 13, 14, 16–18). However, protective mechanisms against oxidative damage may also be involved in this extreme radiation resistance. The lethal effect of ionizing radiation is known to be oxygen enhanced (15) by generating hydrogen peroxide and oxygen free radicals, which damage cell membranes, proteins, and nucleic acids. It has been shown that a pretreatment of *D. radiodurans* at early exponential-growth phase with hydrogen peroxide enhances its resistance to radiation (22). Moreover, *D. radiodurans* expresses relatively high levels of catalase and superoxide dismutase activities (5). However, the importance of the catalase and superoxide dismutase activities in the extreme radiation resistance of *D. radiodurans* has never been tested.

In this study, we have developed a general and simple method to inactivate any targeted gene in *D. radiodurans*. We have successfully constructed both catalase (*katA*) and superoxide dismutase (*sodA*) mutants for investigating their roles in the extreme resistance to ionizing radiation.

Construction of *katA* and *sodA* mutants. The plasmids and strains used in this study are listed in Table 1. Based on the available sequence of the *katA* gene from *D. radiodurans*, three different plasmids were constructed carrying different parts of the gene. A 705-bp DNA fragment from codon 154 to 386 of the deinococcal *katA* gene (accession no. D63898) was PCR amplified with katA-F (5′-GGACTTCGTCGTCAACAACCTC-3′) and katA-B (5′-ATCGGCAGTTGCAGGTAGTTGG-3′) primers. A 2,970-bp PCR fragment containing the complete regulatory and coding regions of *katA* was PCR amplified with katA-F1 (5′-GCTCTTCCATCCCGATCAC-3′) and katA-B6 (5′-CCAGAAAAGCACCGTACTGG-3′) primers. Amplified PCR fragments were blunt end cloned into a pCR-Blunt cloning vector (Km^r; Invitrogen, Carlsbad, Calif.). *D. radiodurans* was transformed with the plasmid constructs, selecting for kanamycin resistance at 25 μg/ml. The transformation protocol was described previously (20). Since pCR-Blunt is a ColE1 plasmid derivative and does not replicate as a plasmid in *D. radiodurans*, kanamycin resistance in *D. radiodurans* was produced by duplication insertion of the plasmid into the chromosome and subsequent amplification of the plasmid (20). To confirm that the plasmid constructs had integrated into the chromosome, high-molecular-weight DNA plugs were prepared and analyzed by pulsed-field gel electrophoresis (PFGE) as described previously (23). DNA plugs were digested with *Not*I restriction enzyme and resolved by PFGE (Fig. 1). Because the pCR-Blunt vector has a unique *Not*I restriction site, its integration into the chromosome creates an extra *Not*I site in the 485-kb *Not*I fragment (Fig. 1A, lane 2) of wild-type R1, yielding 290- and 195-kb fragments (Fig. 1A, lanes 3 to 5). The integration of the plasmid constructs was further confirmed by Southern blot analysis (Fig. 1B) with a nonradioactive probe generated from pKKW1 plasmid DNA by a method described previously (24).

To construct the *sodA* mutant, sequence data of *D. radiodurans* was obtained through early release from The Institute for Genomic Research at www.tigr.org and searched for putative *sodA* sequence. A putative deinococcal *sodA* gene, which shares 57% identity in deduced amino acid sequence with the *Escherichia coli sodA* gene, was identified (data not shown). A 475-bp DNA fragment from codon 24 to 182 of the putative deinococcal *sodA* gene was PCR amplified with SodA-F1 (5′-AGTTGAGGTAGTAGGCGTGTTCCC-3′) and SodA-B3 (5′-GGAAATTCACCACACCAAGCATC-3′) primers. Amplified PCR fragments were blunt end cloned into pCR-Blunt cloning vector. *D. radiodurans* was transformed with the plasmid construct, selecting for kanamycin resistance at 25 μg/ml by using a previously described protocol (20).

Enzymatic activities of *katA* and *sodA* mutants. Transformants with *katA* plasmid constructs (pKKW1, pKKW2, and pKKW3) were tested for catalase activities by adding 10 μl of 3% hydrogen peroxide to 200 μl of overnight culture in a microtiter plate. The robust bubbling due to the formation of oxygen from hydrogen peroxide was an indication of catalase activity. This test indicated that only transformants from plasmid pKKW3 had lost catalase activity. Transformants with a *sodA* plasmid construct (pKKW4) were tested for loss of superoxide dismutase activity by a paraquat sensitivity test with a standardized single filter paper disk method (1). Briefly, 10 μl of 13 mM paraquat was spotted on a 5-mm-diameter sterilized filter paper disk which was placed on a lawn of tested bacteria. Wild-type R1 strain was resistant to paraquat and no zone of

* Corresponding author. Mailing address: Molecular Biosciences, P7-56, Pacific Northwest National Laboratory, P.O. Box 999, Richland, WA 99352. Phone: (509) 376-5097. Fax: (509) 376-6767. E-mail: kk.wong@pnl.gov.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Source
Strain		
D. radiodurans		
R1	Wild type, catalase A and superoxide dismutase positive	ATCC[a]
KKW7001	R1 transformed with pKKW1, catalase A positive	This study
KKW7002	R1 transformed with pKKW2, catalase A positive	This study
KKW7003	R1 transformed with pKKW3, catalase A negative	This study
KKW7004	R1 transformed with pKKW4, superoxide dismutase negative	This study
E. coli Top10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7679 galU galK rpsL*(StrR) *endA1 nupG*	Invitrogen
Plasmids		
pCR-Blunt	pUC derived (Kan^r)	Invitrogen
pKKW1	A 2,970-bp PCR fragment containing the complete regulatory and coding regions of *katA* cloned into pCR-Blunt	This study
pKKW2	A 547-bp *Sac*II fragment (the promoter region of *katA* and part of the coding region) deleted from pKKW1	This study
pKKW3	A 705-bp PCR fragment from codon 154 to 386 of *katA* cloned into pCR-Blunt	This study
pKKW4	A 475-bp PCR fragment from codon 24 to 182 of *sodA* cloned into pCR-Blunt	This study

[a] ATCC, American Type Culture Collection.

growth inhibition was observed, but *sodA* mutants were sensitive to paraquat and a zone of growth inhibition about 25 mm in diameter around the filter disk was observed.

A few selected mutant strains were further confirmed for the loss of catalase or superoxide dismutase activities. Enzymatic activities of the transformants were analyzed by separating total soluble protein extract in a 7.5% nondenaturing gel and staining for catalase activity (Fig. 2) or superoxide dismutase activity (Fig. 3). To prepare the protein extract, *D. radiodurans* cultures were pelleted and resuspended in butanol-saturated phosphate buffer to remove the outer membrane. Cells were washed in 50 mM phosphate buffer (pH 7) and then resuspended again in the same buffer. Cell suspensions were sonicated at 4°C (15 s pulse on, 10 s pulse off) for a total of 5 min by using a Model 60 Sonic Dismembrator set at an intensity of 8 (Fisher Scientific, Pittsburgh, Pa.). Cell debris was removed by centrifugation (13,000 × *g*, 5 min, 4°C), and the cell extracts were either stored on ice or quick frozen at −70°C until subsequent analysis. Protein concentration was determined by using the Bradford protein dye assay (Bio-Rad, Hercules, Calif.) (3). Samples of cell extracts (5 or 10 μg per lane) were separated electrophoretically on 7.5% nondenaturing polyacrylamide gels (22). Gels were stained for catalase activity by the horseradish peroxidase-diaminobenzidine method (6) or stained for superoxide dismutase activity by the nitroblue tetrazolium-riboflavin method (2). As predicted, only transformants derived from the plasmid, which carries part of the internal coding region of the *katA* gene (pKKW3), lost the major catalase activity (Fig. 2). Strain KKW7003 (*katA* mutant) had lost the major catalase, but a minor catalase was still present (Fig. 2). Strain KKW7004 was also confirmed to have lost superoxide dismutase activity (Fig. 3). These results confirmed the validity of the developed targeted mutagenesis method.

This is the first report to demonstrate that duplication insertion (20) can be exploited to create insertional mutations in *D. radiodurans*, although a similar strategy has been used with budding yeast and *Listeria* spp. (9, 25). Unlike the previous direct insertion technique used with *D. radiodurans* (10, 11), which involves complex in vitro DNA manipulation and numerous cloning steps, the current technique is simpler, calling for (i) PCR of the internal fragment of the gene, (ii) a single cloning step to put the PCR fragment into the plasmid, and (iii) transformation of the host. The developed targeted mutagenesis method should be generally applicable for inactivating other genes, thereby facilitating functional studies of targeted genes with unknown function in *D. radiodurans*, whose genome is almost completely sequenced.

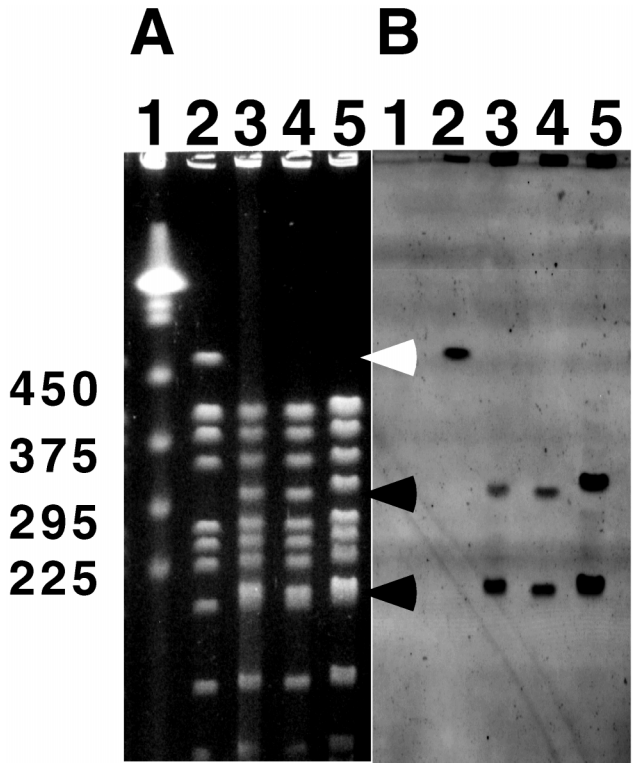

FIG. 1. (A) PFGE analysis of wild type and transformants. DNA agarose plugs were digested with *Not*I restriction enzyme, and digested fragments were resolved with a contour-clamped homogeneous electric field mapper. The gel was stained with ethidium bromide. Lane 1, yeast markers; lane 2, R1 (wild type); lane 3, KKW7001 (complete *katA*); lane 4, KKW7002 (*katA* with its promoter region deleted); lane 5, KKW7003 (partial internal coding region of *katA*). The 485-kb *Not*I fragment (which disappears) is indicated by the white arrowhead. The two new *Not*I fragments are indicated by black arrowheads. The molecular sizes in kilobases are shown on the left. (B) Southern blot from gel shown in panel A and hybridized with fluorecein-labelled probe generated from pKKW1 plasmid DNA. The hybridization signal was detected with a FluorImager SI as described previously (24).

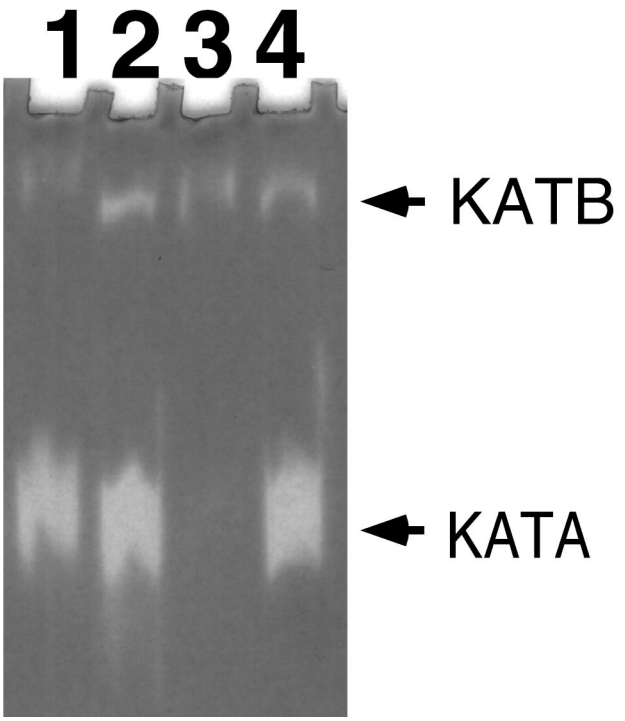

FIG. 2. Analysis of catalase activity. Lane 1, R1 (wild type); lane 2, KKW 7001 (complete *katA*); lane 3, KKW7003 (internal coding region of *katA*); lane 4, KKW7002 (*katA* with promoter region deleted). The two catalase bands shown are labelled KATA (catalase A) and KATB (catalase B).

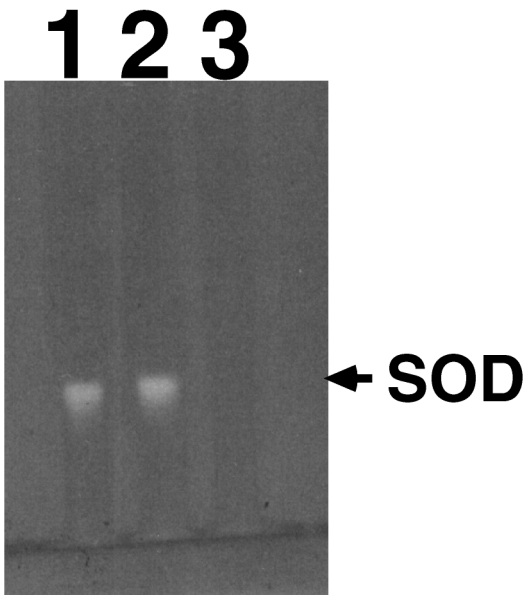

FIG. 3. Analysis of superoxide dismutase activity. Lane 1, R1 (wild type); lane 2, KKW7003 (*katA* mutant); lane 3, KKW7004 (*sodA* mutant). SOD, superoxide dismutase.

Testing ionizing radiation resistance of *katA* and *sodA* mutants. Five-milliliter cultures of wild-type (R1), *katA* mutant (KKW7003), and *sodA* mutant (KKW7004) strains, grown in Falcon 2097 culture tubes to exponential phase at 32°C, were placed on ice inside a 250-ml beaker. All bacterial cultures were simultaneously exposed to ionizing radiation with a dose of 8,000 to 32,000 Gy from a ^{60}Co source irradiator (GammaBeam 650). The *katA* mutant and wild type have similar sensitivities to ionizing radiation when exposed to 8,000 Gy, but the *katA* mutant is reproducibly more sensitive (2- to 15-fold) than the wild-type strain to gamma radiation at doses of 16,000 Gy or higher (Fig. 4). A previous analysis of changes in cellular proteins of *D. radiodurans* after gamma irradiation by two-dimensional gel electrophoresis has identified a number of proteins that increase in amount after irradiation, including a 60-kDa protein, RIP60 (21). The N-terminal end of RIP60, which has been partially determined, has an amino acid sequence of DENNKGV (21), which we were able to match perfectly with the *katA* gene when the amino acid sequence was reverse transcribed into DNA sequence, and the calculated size of deduced *katA* gene product is also 60 kDa (data not shown). Thus, the *katA* gene product is likely the unidentified RIP60 protein that increases in amount after gamma radiation, which is consistent with our finding that *katA* is required for extreme radiation.

On the other hand, the *sodA* mutant is reproducibly more sensitive (3- to 90-fold) than the wild-type strain and the *katA* mutant to ionizing radiation at a dose of 16,000 Gy or higher (Fig. 4). This result suggested that superoxide dismutase encoded by *sodA* is more important than the catalase encoded by *katA* in protecting the bacteria from high doses of ionizing radiation. It is possible that the existence of a secondary catalase B in the *katA* mutant (Fig. 2) is enough to handle most of the hydrogen peroxide generated during ionizing radiation. However, apparently, there does not exist a secondary superoxide dismutase in the *sodA* mutant (Fig. 3) to handle the superoxide anions generated during ionizing radiation. Through analysis of sequence data of *D. radiodurans* obtained through early release from The Institute for Genomic Research, at www.tigr.org, we have identified other genes that encode putative oxidative defense enzymes such as glutathione reductase (*gor*) and alkyl hydroperoxide reductase (*ahpA*). We are currently creating mutations in these genes and constructing mutants with double mutations in order to decipher the oxidative defense systems in *D. radiodurans*.

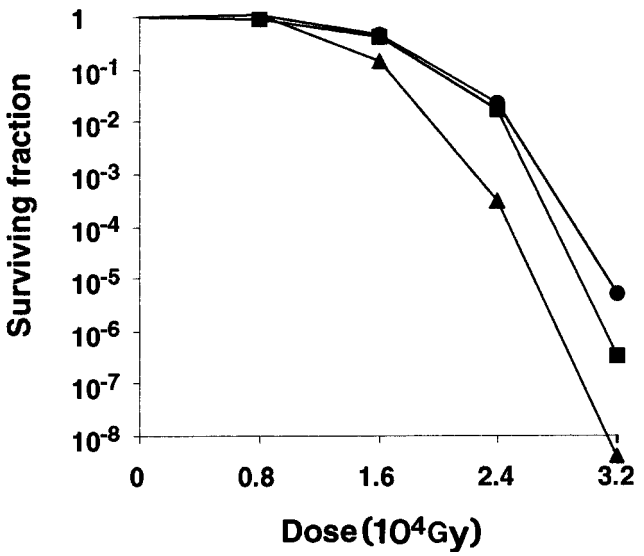

FIG. 4. Cell survival after gamma ray exposure. Gamma radiation was from a ^{60}Co source (GammaBeam 650). Circles, strain R1 (wild type); squares, strain KKW7003 (*katA* mutant); triangles, strain KKW7004 (*sodA* mutant). The exposure rate was 368 Gy per min.

We thank Ken Minton and Rita Cheng for their comments on the manuscript. We are also grateful to Herb Schellhorn for the protocol of catalase activity staining in a nondenaturing polyacrylamide gel and especially to Kevin Groch for operating the ^{60}Co gamma radiation source.

This work was supported by the Department of Energy's Environmental Management Science Program and Microbial Genome Program through a DOE contract, DE-AC06-76RLO 1830, to K.K.W.

REFERENCES

1. **Bauer, A. W., W. M. Kirby, J. C. Sherris, and M. Turck.** 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Patrol. **45:**493–496.
2. **Beauchamp, C., and I. Fridovich.** 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. **44:**276–287.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
4. **Carbonneau, M. A., A. M. Melin, A. Perromat, and M. Clerc.** 1989. The action of free radicals on *Deinococcus radiodurans* carotenoids. Arch. Biochem. Biophys. **275:**244–251.
5. **Chou, F. I., and S. T. Tan.** 1990. Manganese(II) induces cell division and increases in superoxide dismutase and catalase activities in an aging deinococcal culture. J. Bacteriol. **172:**2029–2035.
6. **Clare, D. A., M. N. Duong, D. Darr, F. Archibald, and I. Fridovich.** 1984. Effects of molecular oxygen on the detection of superoxide radical with nitroblue tetrazolium and an activity stain for catalase. Anal. Biochem. **140:**532–537.
7. **Dean, C. J., P. Feldschreiber, and J. T. Lett.** 1966. Repair of x-ray damage to the deoxyribonucleic acid in *Micrococcus radiodurans*. Nature **209:**49–52.
8. **Evans, D. M., and B. E. Moseley.** 1983. Roles of the *uvsC*, *uvsD*, *uvsE*, and *mtcA* genes in the two pyrimidine dimer excision repair pathways of *Deinococcus radiodurans*. J. Bacteriol. **156:**576–583.
9. **Guldener, U., S. Heck, T. Fielder, J. Beinhauer, and J. H. Hegemann.** 1996. A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res. **24:**2519–2524.
10. **Gutman, P. D., J. D. Carroll, C. I. Masters, and K. W. Minton.** 1994. Sequencing, targeted mutagenesis and expression of a *recA* gene required for the extreme radioresistance of *Deinococcus radiodurans*. Gene **141:**31–37.
11. **Gutman, P. D., P. Fuchs, L. Ouyang, and K. W. Minton.** 1993. Identification, sequencing, and targeted mutagenesis of a DNA polymerase gene required for the extreme radioresistance of *Deinococcus radiodurans*. J. Bacteriol. **175:**3581–3590.
12. **Mattimore, V., and J. R. Battista.** 1996. Radioresistance of *Deinococcus radiodurans*: functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation. J. Bacteriol. **178:**633–637.
13. **Minton, K. W.** 1996. Repair of ionizing-radiation damage in the radiation resistant bacterium *Deinococcus radiodurans*. Mutat. Res. **363:**1–7.
14. **Minton, K. W., and M. J. Daly.** 1995. A model for repair of radiation-induced DNA double-strand breaks in the extreme radiophile *Deinococcus radiodurans*. Bioessays **17:**457–464.
15. **Mirsa, H. P., and I. Fridovich.** 1976. Superoxide dismutase and the oxygen enhancement of radiation lethality. Arch. Biochem. Biophys. **176:**577–581.
16. **Moseley, B. E., and H. J. Copland.** 1975. Isolation and properties of a recombination-deficient mutant of *Micrococcus radiodurans*. J. Bacteriol. **121:**422–428.
17. **Moseley, B. E., and D. M. Evans.** 1983. Isolation and properties of strains of *Micrococcus* (*Deinococcus*) *radiodurans* unable to excise ultraviolet light-induced pyrimidine dimers from DNA: evidence for two excision pathways. J. Gen. Microbiol. **129:**2437–2445.
18. **Moseley, B. E., A. Mattingly, and M. Shimmin.** 1972. Isolation and some properties of temperature-sensitive mutants of *Micrococcus radiodurans* defective in DNA synthesis. J. Gen. Microbiol. **70:**399–409.
19. **Setlow, J. K., and M. E. Boling.** 1965. The resistance of *Micrococcus radiodurans* to ultraviolet radiation. II. Action spectra for killing, delay in DNA synthesis, and thymine dimerization. Biochim. Biophys. Acta **108:**259–265.
20. **Smith, M. D., E. Lennon, L. B. McNeil, and K. W. Minton.** 1988. Duplication insertion of drug resistance determinants in the radioresistant bacterium *Deinococcus radiodurans*. J. Bacteriol. **170:**2126–2135.
21. **Tanaka, A., H. Hirano, M. Kikuchi, S. Kitayama, and H. Watanabe.** 1996. Changes in cellular proteins of *Deinococcus radiodurans* following gamma-irradiation. Radiat. Environ. Biophys. **35:**95–99.
22. **Wang, P., and H. E. Schellhorn.** 1995. Induction of resistance to hydrogen peroxide and radiation in *Deinococcus radiodurans*. Can. J. Microbiol. **41:**170–176.
23. **Wong, K. K., and M. McClelland.** 1992. A *Bln*I restriction map of the *Salmonella typhimurium* LT2 genome. J. Bacteriol. **174:**1656–1661.
24. **Wong, K. K., L. C. Stillwell, C. A. Dockery, and J. D. Saffer.** 1996. Use of tagged random hexamer amplification (TRHA) to clone and sequence minute quantities of DNA—application to a 180 kb plasmid isolated from *Sphingomonas* F199. Nucleic Acids Res. **24:**3778–3783.
25. **Wuenscher, M. D., S. Kohler, W. Goebel, and T. Chakraborty.** 1991. Gene disruption by plasmid integration in *Listeria monocytogenes*: insertional inactivation of the listeriolysin determinant *lisA*. Mol. Gen. Genet. **228:**177–182.