Supplementary Materials of

Genome-wide Screening to Identify Responsive Regulators Involved in the Virulence of *Xanthomonas oryzae* pv. *oryzae*

Supplementary Methods

Pathogen inoculation and disease evaluation. *Xoo* strains were cultured for three days on PSA plates containing antibiotics (15 μg/ml cephalexin for PXO99A, 25 μg/ml kanamycin for knock-out mutants, and 10 μg/mL gentamycin for complementary strains) and were suspended to 10^7 cfu (colony forming unit)/ml in water for inoculation using the clipping method (Kauffman et al., 1973). Generally, three healthy, recently and fully developed leaves from each tiller of individuals were chosen for inoculation. Lesion lengths were scored 14 days after inoculation (DAI), and the bacterial population in the inoculated leaves was measured after extraction by colony counting. To collect the bacteria cells from the inoculated leaves, the leaf was detached from the rice, cut into thin slices, suspended, and shaken for 30 min in 10 ml of water containing appropriate antibiotics. After serial dilutions, the supernatant was dropped onto the PSA plate, the plate was cultured at 28°C, and the colonies were counted within three days.

For virulence test, Dong-jin seeds were germinated on petri dishes containing water-drenched filter papers at 28°C for three days, transferred to soil, and then grown in a green house or paddy field before pathogen inoculation. All the inoculation experiments were carried out with six-week-old rice or seven-week-old rice plants (at winter season). Chamber conditions for inoculation test were set at 28°C with 85% humidity for 14 h for days, 25°C and 80% humidity for 10 h for nights.

Generation of knock-out mutant and complementary strains. We first cloned RR genes, amplified by PCR with specific primers (Supplementary Table 1), into pGEM-Teasy vector (Promega) and then inserted the kanamycin cassette into each target gene with appropriate restriction enzymes (Table S1). After confirmation by sequencing, the fragments including target RR genes split by the kanamycin cassette were sub-cloned into pUC18 vector and the knock-out constructs were introduced into PXO99A competent cells by electroporation. Generated knock-out strains were selected on PSA plates containing 25 μg/ml kanamycin and confirmed by PCR as previously reported by Lee et al. (Lee and Ronald, 2007).

To eliminate the polar effect of the RR gene knock-out, we produced the complementary strains continuously expressing target RR proteins with a 6 × His-tag at its N-terminus. Full-length of each RR gene was amplified using the primer set (Table S1), which contained a 6 × His-tag on the forward primer, and cloned into a pGEM-Teasy vector. The 6 × *His-RR* fragment was sequenced and subcloned into the broad-host-range vector pBBR1-MCS5 using EcoRI–BamHI. The resulting construct was introduced into competent cells of each RR knock-out strain by electroporation, and complementary strains were selected on PSA containing 10 μg/ml gentamycin.

Protein extraction and western blot analysis. *Xoo* strains were cultured in 100 ml PSB containing suitable antibiotics until the stationary phase. Cells were harvested by centrifugation (10,000 rpm, 30 min, 4°C), washed with 100 ml distilled water, and harvested again with the same centrifugation conditions. Cell pellets were suspended in 25 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, pH 8.0) containing 20 μl of lysozyme (400,000 U/ml). After incubation overnight at 4°C, cells were sonicated for 5 min (40% AMP, 5 s pulse on, 10 s pulse off) on ice and then centrifuged (12,000 rpm, 30 min, 4°C). The supernatant including total proteins was filtered through a 0.2-μm filter to remove all cell debris. To verify the target protein expression, the total protein extracts were separated by SDS-PAGE and western blot analysis was carried out with His-probe (Novagen) as the primary antibody and HRP-probe (Santa Cruz Biotechnology) as the secondary antibody.

Growth curves of *Xoo* strains in PSB. A single colony of each *Xoo* strain was grown in 5 ml of PSB containing suitable antibiotics. When the cultures reached stationary phase, bacteria strains were added to 50 ml of PSB with 15 μg/ml cephalexin to give a starting population of 10^4 cfu/ml. Growth of the bacteria population was measured by colony counting every 12 hrs for 4 days.

References

Kauffman, H. E., Reddy, A. P. K., Hsieh, S. P. Y. and Merca, S. D. 1973. An improved technique for evaluating resistance of rice varieties to Xanthomonas oryzae. *Plant Dis. Rep.* 57:537-541.

Lee, S. W. and Ronald, P. C. 2007. Marker-exchange mutagenesis and complementation strategies for the Gram-negative bacteria *Xanthomonas oryzae* pv. *oryzae*. *Methods Mol. Biol.* 354:11-18.

Supplementary Table 1. Primer sequences used for cloning of 62 RR genes and restriction enzymes used for insertion of the kanamycin cassette

Gene names	Primer sequence	Gene size (bp)	Enzyme cut position	Product size (Left/Right)	Restriction enzyme
RR1	5′-GAATTCGATGGCTGCAACGAC-3′ 5′-CGTCGCACCCGGGGGCTTTGC-3′ 5′-GCAAAGCCCCGGGTGCGACG-3′	351bp	404bp	833bp (403bp/430bp)	SmaI
RR2	5′-GTGTTCGGCTTCGCGCAATAG-3′ 5′-GTCCAACGGCACCACTTCCT-3′ 5′-TCGTACGTCAGATGCTTAC-3′	1077bp	302bp	983bp (301bp/682bp)	MscI
RR3	5′-TCTAGAGGCGCATTTTCGACGGATAC-3′ 5′-GGTACCGTCACAGGACTGCAGGTCAA-3′	1062bp	735bp, 1238bp	1347bp (391bp/452bp)	XbaI/KpnI
RR4	5′-TCTAGAAAGCTGCATTCCCGTTCCTC-3′ 5′-GGTACCCGACGGCGTGAAGACGTAA-3′	777bp	395bp	753bp (388bp/364bp)	BamHI
RR5	5′-GATCGATCTGGCCATCCTGG-3′ 5′-AGATCGTGCTCTTCGGCAAT-3′	2070bp	664bp, 1081bp	1350bp (506bp/428bp)	BssHII
RR6	5′-CTCCATGGATGACGGAAGGATCAGCCTG-3′ 5′-CTCATATGCCCTGTGGGGTGGCTACGTG-3′	1245bp	89bp, 746bp	1022bp (516bp/506bp)	HincII/PstI
RR7	5′-CAAGCGTCAGTTTGGCTACG-3′ 5′-ACTCAATTGAAGCTGGCCGA-3′	969bp	875bp	902bp (417bp/484)	BssHII
RR8	5′-GTCTAGAGAATTCGCCGCGTTGATTGA-3′ 5′-GGTACCGCGACGGATCATCGATACCA-3′	3396bp	2351bp	1045bp (480bp/563bp)	BamHI
RR9	5′-TCTAGATTATCCGAGGATGCACTGGC-3′ 5′-GGTACCCGTGAGCGTTCATGTGCATT-3′	603bp	288bp	981bp (455bp/527bp)	EcoRV
RR10	5′-GTGGCCGTGAAGGAAGTGCTG-3′ 5′-GAGTCTCGACGGCAGTACTAG-3′	537bp	383bp	885bp (382bp/503bp)	BssHII
RR11	5′-TTCAGGGGGAATGCAATGC-3′ 5′-CGCAACACTCTGTCCCAGAT-3′	1647bp	963bp	1004bp (478bp/1487bp)	BssHII
RR12	5′-CTGCCGCTGTGCGCAAGATCG-3′ 5′-GGTCGCGTTCATCAGGCGGTC-3′	381bp	279bp	663bp (278bp/385bp)	BamHI
RR13	5′-CTCCATGGTGGCACCGCTGACGCTGAAAG-3′ 5′-CTCATATGCCATCACCGCCATCCACTCATC-3′	381bp	300bp	752bp (299bp/453bp)	HincII/SalI
RR14	5′-AGCGATGGCCACGATGTCT-3′ 5′-ACTCATGATTTGCCCGGCA-3′	633bp	406bp	820bp (405bp/415bp)	BssHII
RR15	5′-TCTAGAGCTTTTTGGCGTGAACGTCT-3′ 5′-GGTACCCTTCGGCGACCACTCTCAG-3′	945bp	507bp	842bp (412bp/430bp)	BamHI
RR16	5′-CGTCGCCTTCAGGAGTTTCT-3′ 5′-GGGTAGAGCATCTTGACCCG-3′	1650bp	539bp, 1053bp	1340bp (404bp/423bp)	BssHII
RR17	5′-GTCCTGCTTGTCTTCCACCAC-3′ 5′-GTCAATCGATCGAAACCCGCA-3′	1524bp	870bp 872bp	999bp (525bp/473bp)	BssHII
RR18	5′-GGATCCTCTAGAAACCAGTTGTTCGAAACGGC-3′ 5′-CTCGAGGGTACCTCGATCAACGGCATGTAGGC-3′	192bp	521bp	885bp (428bp/456bp)	EcoRI

Continued

Supplementary Table 1. Primer sequences used for cloning of 62 RR genes and restriction enzymes used for insertion of the kanamycin cassette

Gene names	Primer sequence	Gene size (bp)	Enzyme cut position	Product size (Left/Right)	Restriction enzyme
RR19	5′-CTTGGCCCAACGGTTAATGC-3′ 5′-TTCACGGAGATAGGCTGCAC-3′	348bp	573bp, 787bp	986bp (389bp/391bp)	BssHII
RR20	5′-GGCCTGACTTACGAGACCTG-3′ 5′-GTAGGGCTTTTCCAGGAAGG-3′	330bp	334bp	551bp (333bp/218bp)	HincII/PstI
RR21	5′-CGGCTTGGTCAGATATTGCT-3′ 5′-GCGGGAAGGTTGTGAAGTAG-3′	402bp	578bp	967bp (577bp/390bp)	PstI
RR22	5′-CCCTCCAGGAACTGCTTCAG-3′ 5′-AAAATTGCGGACCAGCAACC-3′	363bp	766bp, 190bp	903bp (442bp/521bp)	MluI
RR23	5′-GAATGGATCACCGAGCATGG-3′ 5′-TCTCTCCATCATCGACGTCG-3′	3576bp	1002bp	1542bp (1001bp/541bp)	MscI
RR24	5′-GCCTTGGCTTTCAAAAATGG-3′ 5′-CACAAGGTGCGATCTGGATA-3′	3558bp	321bp	903bp (320bp/583bp)	SmaI
RR25	5′-CGTTGGCCCAAGCGTCTCC-3′ 5′-GCCGAGTGTTGCCAGGAACC-3′	2766bp	392bp	784bp (391bp/393bp)	BssHII
RR26	5′-ATGAACATGGCCACATGCCC-3′ 5′-TCAATGCAGCAACGCAGCCA-3′	723bp	836bp	915bp (835bp/80bp)	PvuII
RR27	5′-CTGCGGCCGCACCGGTATTGGCCGCAGCC-3′ 5′-CTCATATGTCGCTAGGATGGCACGCGTC-3′	792bp	44bp, 641bp	1000bp (500bp/500bp)	HincII/PstI
RR28	5′-AGCCTCTTCCGACCGTAG-3′ 5′-GCGACAACAGTCAGGCATC-3′	678bp	388bp	1001bp (387bp/614bp)	MscI
RR29	5′-TGCCAAGCTAGTGAGCAGAC-3′ 5′-CGTGCATCGAACATCTCACC-3′	699bp	438bp	850bp (437bp/413bp)	BamHI
RR30	5′-GAAGCTTCTGTTCCGTTTCCCACAGGA-3′ 5′-GTCTAGAGAACGCAGACCGATGGGAT-3′	2646bp	1663bp	974bp (480bp/493bp)	SalI
RR31	5′-TCACTCAGGACGAACTGCAC-3′ 5′-GCTGGAAGCACGCAGATTTC-3′	657bp	766bp, 946bp	1099bp (473bp/447bp)	MscI
RR32	5′-CAGGTGCATGTCTGAATCACG-3′ 5′-CTAGATACAGCAGCAAGGTGC-3′	702bp	480bp	998bp (479bp/519bp)	HincII
RR33	5′-CGGATTTATCCACCCGCTGA-3′ 5′-ATGGAAGGCGTTAGCACGAA-3′	690bp	858bp	964bp (408bp/470bp)	BssHII
RR34	5′-CTGACACCGGAAGTTCTGCT-3′ 5′-CTCAGCAAGTCCCAGCAAC-3′	1116bp	390bp	543bp (352bp/191bp)	BssHII
RR35	5′-CTGCAGCGGGTTTCAGGCAGAGAAGT-3′ 5′-GAGCTCGTCGAATTGAAGGTGCCAGC-3′	387bp	740bp	1010bp (559bp/451bp)	SalI
RR36	5′-GAAGCTTGAATAGACGCGCGGCATTGA-3′ 5′-GTCTAGAGCACGTTCTGGTCGTTGT-3′	3453bp	1489bp	937bp (452bp/484bp)	SalI
RR37	5′-GCAGATTCTTTTCGGCGGTG-3′ 5′-AACCGCTGAACGTAGACAGT-3′	672bp	566bp	947bp (534bp/412bp)	BssHII
RR38	5′-GTGAGCGCGACCATCATTCC-3′ 5′-TCAGAGCACCGCGCTCGTGA -3′	1827bp	119bp 447bp	635bp (355bp/280bp)	SfoI
RR39	5′-CTGCAGTGGTTGAACTTGCCACCGAT-3′ 5′-GGATCCAGATAACGCAGCAGCGACTT-3′	1280bp	543bp	937bp (487bp/451bp)	BssHII

Continued

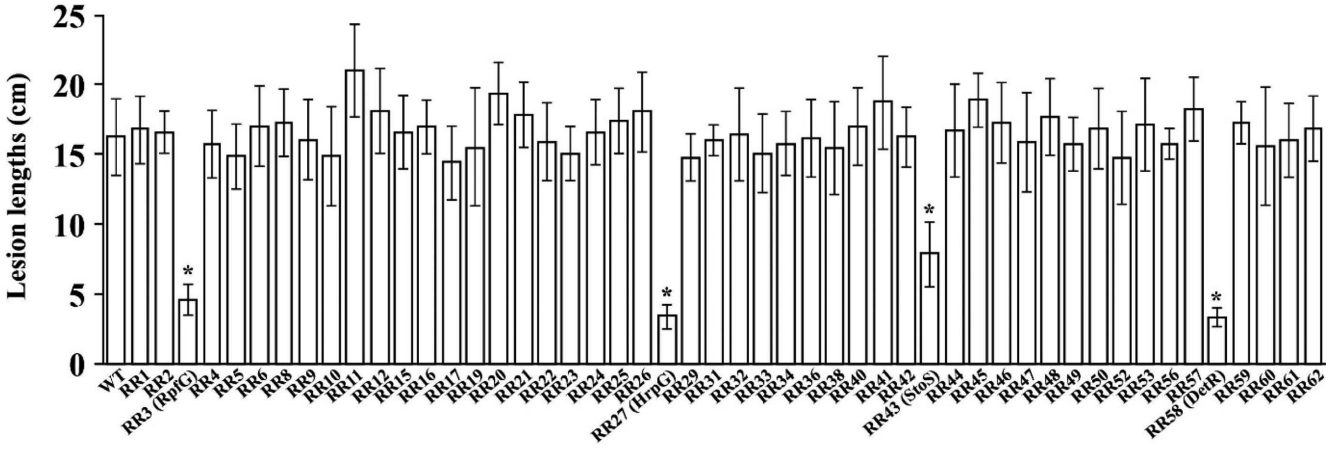
Supplementary Table 1. Primer sequences used for cloning of 62 RR genes and restriction enzymes used for insertion of the kanamycin cassette

Gene names	Primer sequence	Gene size (bp)	Enzyme cut position	Product size (Left/Right)	Restriction enzyme
RR40	5′-CCTCGTCCAGCAACTGGC -3′ 5′-GAACGTCCCGGCGATACTG -3′	381bp	650bp	833bp (547bp/258bp)	BssHII
RR41	5′-GAACAGGCTCATGAACGGCT-3′ 5′-GCCATCGTGTCCAATTTCCC-3′	498bp	569bp	892bp (352bp/520bp)	BssHII
RR42	5′-GACACCGTCGGCCCGTCACC-3′ 5′-TGTCGGTGGTGCAAAGCATC-3′	729bp	96bp	244bp (119bp/125bp)	SmaI
RR43	5′-GAAGCTTCCCTTGTAGGTGGTGTGTCC-3′ 5′-GTCTAGACACAACATCGATGCCGGTTT-3′	2037bp	1491bp	902bp (463bp/438bp)	HincII
RR44	5′-CATCTGCAGCACGCGAAATTG-3′ 5′-GACCTGCTGTTGATGGACCTC-3′	669bp	179bp 276bp	892bp (447bp/445bp)	BssHII
RR45	5′-TCTAGAGCCATACCGTTTTCACCCTG-3′ 5′-GGTACCTTGACGCAGGTCTTCCAACG-3′	1395bp	512bp, 787bp	1131bp (367bp/488bp)	BssHII
RR46	5′-TCCACCAGATCTCAAACACC-3′ 5′-GTGACCGCCTGATCCTCCAG-3′	795bp	184bp 681bp	917bp (542bp/375bp)	BssHII
RR47	5′-CACTGGGCGGTTGACGCATGG-3′ 5′-CATGCAGATCCTGTCGCCGCAC-3′	708bp	556bp	635bp (555bp/80bp)	SmaI
RR48	5′-ACTGGATCATCTGTTTTTCC-3′ 5′-ATCATCTGGTCAAGCCGGTG-3′	1347bp	399bp	830bp (398bp/432bp)	BssHII
RR49	5′-CTCCATGGCAGCCGCTGGTCGAAAATGC-3′ 5′-CTCATATGCGGAGCGTGGTCATTCCAGC-3′	738bp	401bp	900bp (400bp/500bp)	HincII
RR50	5′-TCTAGACAATGCCGTCAAGTACAGCG-3′ 5′-GGATCCATGCTCAGATCCGACAGCAC-3′	441bp	896bp	965bp (448bp/516bp)	NcoI
RR51	5′-TCTAGAGCCGAATCGATGTTGTCGTT-3′ 5′-GGTACCTTTGCGCAATACATGCACCG-3′	1503bp	1133bp	840bp (526bp/313bp)	MscI
RR52	5′-ACCCGCCGCCTGCAGCGTTAC-3′ 5′-GTGCTTGAGCAGCAGTGCGAGC-3′	717bp	485bp	964bp (484bp/480bp)	HincII
RR53	5′-GTCGATGGCGCGGTCGTAGACG-3′ 5′-ACGTGGCGGAGATGCGCTCTG-3′	720bp	585bp	954bp (584bp/370bp)	HincII
RR54	5′-CGCATACGCTGGCCAAATTGT-3′ 5′-ACCGCAGGATCTCGGTCTCG-3′	642bp	497bp	959bp (496bp/463bp)	HincII
RR55	5′-CTCCATGGTTTTTCGGCGTACAACTCGC-3′ 5′-CTCATATGTCGGAATTCAGCAACGAGCG-3′	717bp	64bp, 356bp	1000bp (500bp/500bp)	BssHII
RR56	5′-TCTAGACTCGAAGGTGTCCAGCAGTT-3′ 5′-GGTACCAACGCGCTCAAGTTCAATCG-3′	831bp	539bp, 724bp	1064bp (400bp/400bp)	BssHII
RR57	5′-CGAACCGCAGCACAGTGAACC-3′ 5′-TGCAATCCCACGAGGCAATCG-3′	465bp	466bp	919bp (465bp/454bp)	HincII
RR58	5′-GCGCTGGAGTCGCGTAATTCGC-3′ 5′-CCTGCTCTACCTGCAACGCAGC-3′	690bp	466bp	760bp (465bp/296bp)	BamHI
RR59	5′-TGATGCAACTCAGCCGCAGC-3′ 5′-CCTTCGGCAACGGCAGTGAG-3′	1284bp	404bp	790bp (403bp/387bp)	BssHII
RR60	5′-CCGCAGGCGTATCTCAAACG-3′ 5′-GCGGTGAGCACTTCTTCCAGG-3′	1122bp	465bp	955bp (464bp/491bp)	EcoRV
RR61	5′-GCGCTGGAGTCGCGTAATTCGC-3′ 5′-CCTGCTCTACCTGCAACGCAGC-3′	690bp	466bp	761bp (465bp/296bp)	BamHI
RR62	5′-TCTTGTTCCAGCGGTTTTCT-3′ 5′-CGAAGACGACCAGATCAGC-3′	1401bp	549bp	995bp (548bp/447bp)	HincII

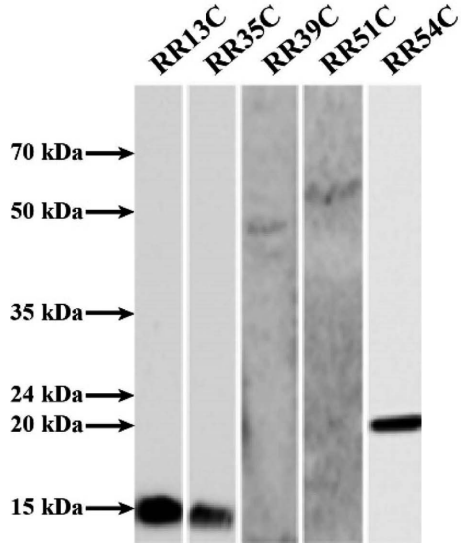
Supplementary Table 2. Complementary strains used in this study

Strains	Locus tag No. of mutated gene	Characteristics	Source
RR13C	PXO_RS11975/ PXO_RS12905	Km^r, Gm^r, RR13 complemented with pBBR1-MCS5 inserted by *6XHis-PXO_RS11975*	in this study
RR35C	PXO_RS20535	Km^r, Gm^r, RR35 complemented with pBBR1-MCS5 inserted by *6XHis-PXO_RS20535*	in this study
RR39C	PXO_RS21800	Km^r, Gm^r, RR39 complemented with pBBR1-MCS5 inserted by *6XHis-PXO_RS21800*	in this study
RR51C	PXO_RS04305	Km^r, Gm^r, RR51 complemented with pBBR1-MCS5 inserted by *6XHis-PXO_RS04305*	in this study
RR54C	PXO_RS06090	Km^r, Gm^r, RR54 complemented with pBBR1-MCS5 inserted by *6XHis-PXO_RS06090*	in this study

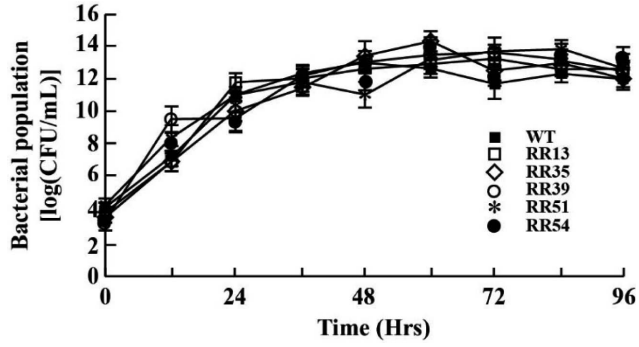
Supplementary Fig. 1. Virulence phenotypes of PXO99A (WT), RR13, RR35, RR39, RR51 and RR54 strains.



Supplementary Fig. 2. Inoculation test with PXO99A and RR mutant strains. A high population density of each mutant strain was inoculated on leaves of Dong-jin rice plants, and the lesion lengths were scored at 14 days after inoculation. Each strain was cultured on PSA containing cephalexin (for PXO99A) and kanamycin (for mutant strains) at 28°C for 3 days. The bacteria were suspended in distilled water to achieve a population of 10^7 cfu/ml and inoculated using the clipping method. To prepare the rice plants, Dong-jin seeds were germinated in water at 28°C for 4 days and then planted into soil and grown for 6 weeks in a greenhouse. The 6-week-old plants were transferred to a growth chamber at least 2 days prior to inoculation. The chamber conditions were as follows: 14/10 h of light/dark at 28/25°C and 80/90% relative humidity. * indicates that the lesion length of mutant strains was significantly different from that of PXO99A by Duncan's test ($P < 0.05$). The virulence tests were repeated three times with high consistency. The results from one experiment are shown.



Supplementary Fig. 3. Western blot analysis of the complementary strains RR13C, RR35C, RR39C, RR51C, and RR54C, which continuously express 6×His-PXO_RS11975, -PXO_RS20535, -PXO_RS21800, -PXO_RS04305, and -PXO_RS06090 recombinant proteins, respectively. Bacteria were harvested at stationary phase. Total proteins were extracted from 100 ml of culture by sonication, and western blot analysis following SDS-PAGE was carried out with His-probe (Novagen) as the primary antibody and HRP-probe (Santa Cruz Biotechnology) as the secondary antibody.



Supplementary Fig. 4. Growth curves of PXO99A, RR13, RR35, RR39, RR51, and RR54 in PSB. Bacteria were cultured in 50 ml PSB with an initial population of approximately 10^4 cfu/ml. Samples were collected every 12 h to count the bacterial population. The experiments were repeated three times with the same result, and the results from one experiment are presented.