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MarA-Like Regulator of Multidrug Resistance in *Yersinia pestis*

Rupa A. Udani[1]† and Stuart B. Levy[1,2]*

Center of Adaptation Genetics and Drug Resistance and Department of Molecular Biology and Microbiology[1] and Department of Medicine,[2] Tufts University School of Medicine, Boston, Massachusetts 02111

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MarA47$_{Yp}$ from *Yersinia pestis*, showing 47% identity to *Escherichia coli* MarA in its N terminus, caused resistance to antibiotics and to organic solvents when expressed in both *E. coli* and *Y. pestis*. Resistance was linked to increased expression of the AcrAB multidrug efflux pump. In four of five spontaneous multidrug-resistant mutants of *Y. pestis* independently selected by growth on tetracycline, the *marA47*$_{Yp}$ gene was overexpressed. The findings suggest that *marA47*$_{Yp}$ is a *marA* ortholog in *Y. pestis*.

Multiple-drug resistance in microorganisms is commonly acquired through plasmids, transposons, or integrons specifying different genes for resistance (12). Alternatively, mutations in chromosomal genes may produce resistance to a wide variety of antibiotics and other toxic substances. One such chromosomal locus, the *mar* (multiple antibiotic resistance) locus in *Escherichia coli* and other enteric bacteria, results in resistance to multiple antibiotics, oxidative stress agents, organic solvents, and disinfectant products (1, 2, 6, 7, 10, 27, 29).

In *E. coli*, the *mar* locus encodes the *marRAB* operon (5), specifying MarR, which represses the operon by binding to *marO* (15), and MarA, which positively regulates the operon and affects expression of the Mar phenotype and many other chromosomal genes through both activation and repression (4, 16, 21, 24–26). *marB* specifies a small putative protein of unknown function (5). Multidrug resistance is principally caused by MarA-mediated overexpression of the *acrAB* efflux system (19).

E. coli MarA is a member of the XylS/AraC family of transcriptional activators which contain two helix-turn-helix motifs (9). Most larger members (>250 amino acids [aa]) also possess an effector domain at either the N or C terminus of the protein (9). MarA, a 129-amino-acid protein, lacks the effector domain. MarA control of other chromosomal genes occurs via binding to the "marbox," a 20-bp degenerate MarA binding site (3, 11).

The genus *Yersinia* contains 11 species, of which 3 are human pathogens: *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* (20). *Yersinia pestis* is the causative agent of bubonic and pneumonic plague. The majority of *Y. pestis* strains contain three plasmids involved in virulence, as well as the ~102-kb chromosomal pathogenicity locus *pgm*, specifying genes for a *Yersinia*-specific iron siderophore and its receptor and hemin adsorption (20). The report of a multidrug-resistant strain (8) and the possible use of *Y. pestis* as a vehicle for biological warfare have caused increased public health concern.

We asked whether *Y. pestis*, like other members of the *Enterobacteriaceae*, contained chromosomal loci, such as *marA*, involved in multidrug resistance. We identified a *Yersinia* ortholog of MarA, gene YPO2243 (designated *marA47*$_{Yp}$), which produced multidrug resistance in *E. coli* and in *Y. pestis* when overexpressed in each host.

* Corresponding author. Mailing address: Center for Adaptation Genetics and Drug Resistance, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Phone: (617) 636-6764. Fax: (617) 636-0458. E-mail: stuart.levy@tufts.edu.

† Present address: Prodesse, Inc., W229 N1870 Westwood Dr., Waukesha, WI 53186.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Table 1 shows the bacterial strains and plasmids used in this study. The *Y. pestis* strain without the *pgm* pathogenicity locus was approved by the University Institutional Biosafety Committee for studies at the BL2 level. The *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth. The *Y. pestis* strains were grown in brain heart infusion broth (BHI) (Difco) at 26°C and BHI with 5 mM $CaCl_2$ at 37°C. Ampicillin (100 μg/ml), kanamycin (50 μg/ml), and tetracycline (5 μg/ml) were added when necessary. The plasmid pJP105 (22) was used to clone the gene *marA47*$_{Yp}$ behind the *lac* promoter, regulated by the plasmid-borne *lac* repressor. The plasmid pMB102 (21) bearing *E. coli marA* was used as a positive control. The *lacZ* promoter was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.2 mM. pJPBH was the vector control (24).

Cloning of *marA47*$_{Yp}$. The primers A47YPF (CCCAAGCTTGGGAATAAA ATGATGAGTGAAGACATATTG) and A447YPR (CGGGATCCCGGCGTT AATTCACTGCCATCA) were used at a 50-pmol final concentration to amplify the *marA47*$_{Yp}$ coding region beginning with the first putative ATG. The PCR used the *Taq* polymerase (Invitrogen) and conditions recommended by the manufacturer. *marA47*$_{Yp}$ was directionally cloned into pJP105 between the BamHI and HindIII sites using a 1:1 vector to insert ratio and T4 DNA ligase (New England Biolabs), creating the plasmid pRU1.

Drug susceptibility testing. Drug susceptibilities of strains were determined using E-tests (gift of AB Biodisk, Solna, Sweden). The *E. coli* strains were tested by incubation at 37°C overnight on LB agar plates with or without IPTG. *Y. pestis* was examined after incubation for 2 days on BHI agar plates for growth at 26°C or on BHI agar plates with 5 mM $CaCl_2$ for growth at 37°C with or without IPTG.

Organic solvent tolerance. An overnight culture was diluted 1/100 in respective medium (LB or BHI) containing 10 mM $MgSO_4$ and grown at either 37°C or 26°C to an A_{530} of 0.2. After 10-fold dilutions with phosphate-buffered saline, 5 μl was spotted onto agar plates containing 10 mM $MgSO_4$ and allowed to dry. The plates were flooded with 6 to 8 ml of organic solvent (hexane, cyclohexane, and pentane) under a fume hood and placed in a sealed container. The *E. coli* cultures were incubated at 30°C overnight and the *Y. pestis* at room temperature for 2 days.

Isolation of *Y. pestis* spontaneous mutants. Approximately 4×10^4 cells of an overnight culture of *Y. pestis* Ev7651F were inoculated into 15 tubes of fresh BHI broth (2 ml) and incubated at 26°C overnight to an A_{600} of 0.7 to 0.8. One hundred fifty microliters from each tube was plated on BHI agar plates containing 5 μg/ml tetracycline and incubated at 26°C. Over 4 to 5 days, newly appearing colonies were marked on each day. Approximately 75 mutants growing on tetracycline plates were obtained. Five were selected for further study.

RT-PCR. Early-log-phase cultures of *Y. pestis* EV7651F/RU1, grown at 26°C and 37°C, were further grown in the presence or absence of 0.2 mM IPTG for ~3 h. RNA from the spontaneous mutants was isolated from cultures grown at both 26°C and 37°C without IPTG. RNA isolated using a QIAGEN RNA isolation kit

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristic(s)	Reference
E. coli		
AG100	*uncB*$^+$ *argE3 thi-1 rpsL xyl mtl galK supE44*	10
AG112	Spontaneous *marR* mutant of AG100	18
AG100B	AG100 *acrR* replaced with kanamycin cassette	19
AG100Kan	AG100 *marCORAB* replaced with kanamycin cassette	13
AG100A	AG100 *acrAB* replaced with kanamycin cassette	19
N8453	Δ*lacU169* strain GC4468 deleted of *mar* (Δ39 kb), *soxS*, and *rob*; chloramphenicol resistant (Tn*9*)	16
Y. pestis		
EV7651F	Parental *Y. pestis* strain with deleted *pgm* locus; contains 70-kb virulence plasmid	17
Plasmids		
pJP105	pBR322 *lacI lacZp*::*soxS* (tetracycline susceptible)	22
pMB102	*E. coli marA* replacing *soxS* in pJP105 between BamHI and HindIII	21
pRU1	$marA47_{Yp}$ cloned into pJP105 using BamHI and HindIII sites	This study
pJPBH	pJP105 digested with BamHI and HindIII to eliminate *soxS*, followed by blunt ending and religation	24

was DNase treated. Reverse transcription (RT)-PCRs were performed using ~500 ng of total RNA. The RT reaction was performed using the Superscript III first-strand synthesis system (Invitrogen) and following the manufacturer's protocol. A minus RT enzyme reaction of each sample served as a negative control.

Real-time PCR. The real-time PCR assay was carried out using the Quantitect SYBR Green PCR kit (QIAGEN). The reactions followed the kits' protocol. The primers either to $acrAB_{Yp}$ (SACABF-GCCGGTGATCG CCTGATTA and SACABR-ATGGTCGGATATTGCGCTAC), $marA47_{Yp}$ (SA47F-CTATATCCGTGGGCGAGTGT and SA47R-GCTTGATTTCC GGCGTATAA), or YP16s (S16VF-CAGAAGAAGCACCGGCTAAC and S16VR-CGGGGATTTCACATCTGACT) rRNA were used for the real-time PCR carried out in the MX4000 multiplex quantitative PCR system from Stratagene. The annealing temperature for the reactions was 55°C for 30 s. Three individual readings were taken at annealing temperature during plateau and dissociation analysis. Each reaction was performed at least twice. The amount of 16S rRNA from each strain was used to normalize mRNA levels. The C_T (threshold cycle) of each gene from the amplification plot was used to calculate the ΔC_T ($\Delta C_T = C_T$ of gene X of parental EV7651F minus C_T of gene X of the mutant). The quantity of gene expression was given by $q = 2\Delta^{C_T}$. The degree of difference in expression of gene X with respect to 16S rRNA was calculated to determine the relative expression of gene X in the mutant compared to that in the wild type.

Western blot analysis. Protein extracts of *E. coli* and *Y. pestis* strains were separated by gel electrophoresis and blotted to membranes for Western analysis using anti-*E. coli* AcrA antibody (gift of H. Zgurskaya, University of Oklahoma), following published methodology (28).

RESULTS AND DISCUSSION

Search for MarA and AcrAB orthologs in *Y. pestis* genome. A *marRAB* locus similar to that in *E. coli* was not found in the genome sequence of *Y. pestis* CO92 (www.sanger.ac.uk), but a number of coding sequences showing homology to the individual genes, *marR* and *marA*, were detected. Genes for four orthologs of the MarA protein were found on the chromosome and one each on the 70-kb virulence and the 100-kb plasmids of *Y. pestis*. Orthologs of the *E. coli* membrane efflux pumps AcrAB, AcrEF, and EmrEF were also identified using the respective proteins as query sequences.

Cloning of the *marA* ortholog of *Y. pestis*. The attenuated *Y. pestis* strain EV7651F was used to clone potential *marA* orthologs. The coding sequence for YPO1737 (36% identity to *E. coli* MarA) was first chosen because of its size similarity (128 aa) to the *E. coli* MarA protein. The gene, cloned into plasmid pJP105 downstream of the IPTG-inducible promoter, did not produce any antibiotic resistance in *E. coli* AG100 or *Y. pestis* EV7651F when induced by IPTG (data not shown). This lack of activity of a putative MarA homolog was reported earlier for *E. coli ykgA*, which did not produce multidrug resistance when overexpressed in *E. coli* (14).

The product of the YPO2243 coding sequence (which we designated $marA47_{Yp}$), though double in size (297 aa), was next chosen because of the relatively high identity of its N-terminal half to *E. coli* MarA (47%) and SoxS (46%) and comparatively lower identity to *E. coli* Rob (32%) (which is similar in size). The $MarA47_{Yp}$ protein has the two helix-turn-helix motifs at its N terminus rather than the C terminus as found for AraC. Figure 1 shows the alignment of the N-terminal region of the $MarA47_{Yp}$ protein with *E. coli* MarA and other related proteins using the ClustalW program. The phylogenetic tree analysis of proteins examined in Fig. 1 places $MarA47_{Yp}$ next to *E. coli* MarA (data not shown).

***marA47*$_{Yp}$ expression results in multiple drug resistance and organic solvent tolerance in both *E. coli* and *Y. pestis*.** Plasmid RU1, bearing the cloned $marA47_{Yp}$ gene, was transformed into *E. coli* and *Y. pestis* hosts. When induced by IPTG, $marA47_{Yp}$ produced multiple drug resistance and organic solvent tolerance in AG100 (Table 2) with values similar to those for AG100 bearing *E. coli marA* on the same plasmid vector (pMB102). The vector pJPBH served as a negative control. In addition, *E. coli* strain AG112, which constitutively expresses *marA* because of an inactivating mutation in *marR*, also expressed multidrug resistance as expected. Neither transformant showed resistance to aminoglycosides.

The induction of $marA47_{Yp}$ was also studied in the *marRAB*-deleted mutant AG100Kan and in a *mar soxS rob* triple deletion mutant, *E. coli* N8453. The expression of $marA47_{Yp}$ in both AG100K and N8453 resulted in resistance to multiple antibiotics and organic solvent tolerance (Table 2) comparable to that observed with cloned *E. coli marA* in AG100Kan and N8453 (Table 2). Thus, $marA47_{Yp}$ can function independently of the intrinsic *mar*, *soxS*, and *rob* genes of *E. coli*.

In *Y. pestis* EV7651 IPTG induction of $marA47_{Yp}$ on RU1 led to multidrug resistance at both 26°C (the temperature at which *Yersinia* grows in fleas) and also at 37°C (temperature

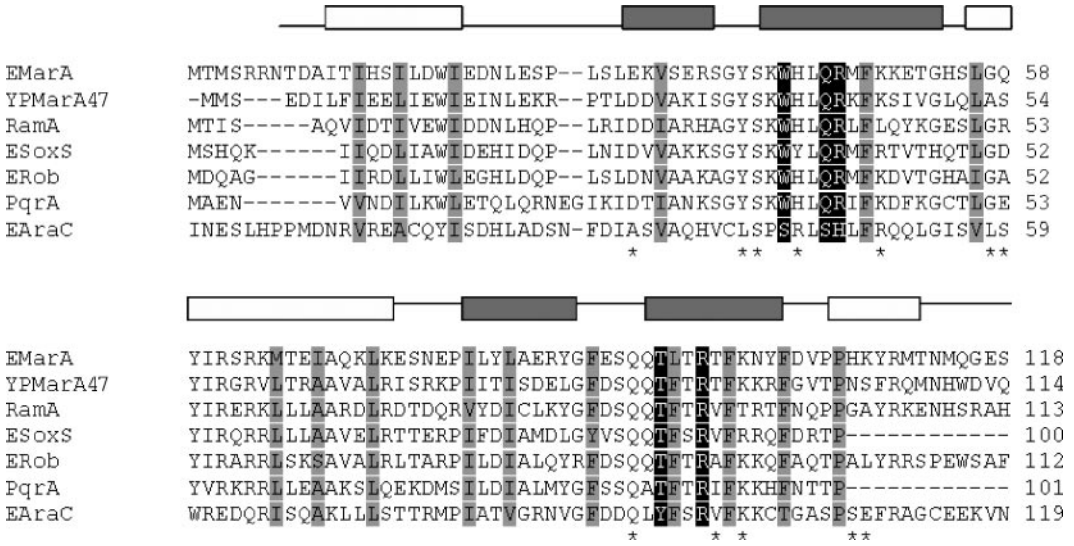

FIG. 1. Alignment of MarA47$_{Yp}$ with MarA and other members of the AraC family. The ClustalW program was used for the alignment of MarA47$_{Yp}$ with other members of AraC family. The shaded and open horizontal boxes represent the helix-turn-helix (HTH) motifs of MarA. The gray shaded residues are the hydrophobic core of the HTH motif, and the black shaded residues determine the sequence specificity. Asterisks mark the residues that interact with the phosphate backbone group of DNA as determined from the structure of *E. coli* MarA (23). EMarA, *E. coli* MarA; YPMarA47, *Y. pestis* ortholog of MarA; RamA, MarA ortholog from *Klebsiella pneumoniae*; ESoxS, *E. coli* SoxS; ERob, *E. coli* Rob; PqrA, MarA ortholog from *Proteus vulgaris*; EAraC, *E. coli* AraC.

in mammalian hosts) (Table 3). Growth under the organic solvent hexane was also noted only in the presence of 0.2 mM IPTG (data not shown). Transformants of EV7651F bearing the vector alone showed no drug or organic solvent resistance.

The *marA47*$_{Yp}$-associated multidrug resistance functions via the AcrAB efflux pump. Cloned *marA47*$_{Yp}$ on pRU1 transformed into *E. coli* AG100A (*acrAB* deletion mutant) neither conferred multidrug resistance nor exhibited tolerance to organic solvents. Western blot analysis of AG100/RU1, AG100Kan/RU1, and N8453/RU1 cell extracts showed increased expression of AcrA only in the presence of IPTG induction of *marA47*$_{Yp}$ (data not shown). These results imply that MarA47$_{Yp}$, like MarA, confers multiple drug resistance through the AcrAB efflux pump in *E. coli*.

To determine whether the induction of *marA47*$_{Yp}$ in

TABLE 2. Drug susceptibilities and organic solvent tolerances of *E. coli* strains with or without *marA* or *marA47*$_{Yp}$

Strain[a]	MIC of drug (μg/ml)[b]												Growth on solvent[c]					
	Tet		Dox		Rif		Chl		Nor		Nal		Hexane		Cyclohexane		Pentane	
	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
AG100	1.0	1.0	1.5	1.5	7.5	7.5	4.0	4.0	.08	.08	3.5	3.5	**+++**	**+++**	−	−	−	−
AG112	**6.0**	**6.0**	**6.0**	**6.0**	**9.0**	**9.0**	**10**	**10**	**.25**	**0.25**	**6.0**	**6.0**	+++	+++	+++	+++	++	++
AG100/v	1.0	1.0	1.5	1.5	7.5	7.5	4.0	4.0	.08	.08	3.5	3.5	**+++**	**+++**	−	−	−	−
AG100/102	1.0	**2.0**	1.5	**2.5**	7.5	8.0	4.0	**6.0**	.08	**0.15**	3.5	**4.5**	+++	+++	−	+++	−	−
AG100/RU1	1.0	**2.0**	1.5	**2.5**	7.5	8.0	4.0	**6.0**	.08	**0.15**	3.5	**4.5**	+++	+++	−	+++	−	+
AG100Kan	1.0	1.0	1.5	1.5	7.5	7.5	4.0	4.0	.08	.08	3.0	3.0	**+++**	**+++**	−	−	−	−
AG100Kan/v	1.0	1.0	1.5	1.5	7.5	7.5	4.0	4.0	.08	.08	3.0	3.0	**+++**	**+++**	−	−	−	−
AG100Kan/102	1.0	**2.0**	1.5	**2.5**	7.5	8.0	4.0	**5.0**	.08	**0.15**	3.0	**4.0**	+++	+++	−	++	−	−
AG100Kan/RU1	1.0	**2.0**	1.5	**2.5**	7.5	8.0	4.0	**5.0**	.08	**0.15**	3.0	**4.0**	+++	+++	−	++	−	+
AG100A	0.3	0.3	0.3	0.3	6.0	6.0	1.5	1.5	ND	ND	ND	ND	−	−	−	−	−	−
AG100A/v	0.3	0.3	0.3	0.3	6.0	6.0	1.5	1.5	ND	ND	ND	ND	−	−	−	−	−	−
AG100A/102	0.3	0.3	0.3	0.3	6.0	6.0	1.5	1.5	ND	ND	ND	ND	−	−	−	−	−	−
AG100A/RU1	0.3	0.3	0.3	0.3	6.0	6.0	1.5	1.5	ND	ND	ND	ND	−	−	−	−	−	−
N8453	0.5	0.5	0.6	0.6	7.0	7.0	ND	ND	.01	0.04	3.0	3.0	−	−	−	−	−	−
N8453/v	0.5	0.5	0.6	0.6	7.0	7.0	ND	ND	.01	0.04	3.0	3.0	−	−	−	−	−	−
N8453/102	0.5	**0.8**	0.6	**1.5**	7.0	8.0	ND	ND	.01	0.05	3.0	**4.0**	−	+++	−	+	−	−
N8453/RU1	0.5	**1.0**	0.6	**1.6**	7.0	8.0	ND	ND	.01	0.05	3.0	**4.0**	−	+++	−	++	−	+

[a] AG100, wild type; AG112, *marR* mutant; AG100Kan, Δ*mar* locus; AG100A, Δ*acrAB*; N8453, Δ*mar*, *sox rob*; 102, plasmid pMB102; RU1, plasmid pRU1; v, pJPBH (vector control). See Table 1 for strain descriptions. N8453 has a chloramphenicol resistance transposon.

[b] Minus or plus sign, absence or presence of 0.2 mM IPTG; Tet, tetracycline; Dox, doxycycline; Rif, rifampicin; Chl, chloramphenicol; Nor, norfloxacin; Nal, nalidixic acid; ND, not determined. Reproducibly observed increased MICs (compared to those for parental strains) are in boldface. Results are averages for experiments performed in triplicate.

[c] Growth compared to growth on medium without organic solvents: −, no growth; +, minimal growth; ++, moderate growth; +++, full growth.

TABLE 3. Drug susceptibilities of *Y. pestis* EV7651F with vector or pRU1 at 26°C and 37°C (with 5 mM $CaCl_2$)[a]

Antibiotic	Treatment with IPTG (0.2mM)	MIC of drug			
		EV7651F/v		EV7651F/RU1	
		26°C	37°C	26°C	37°C
Tet	−	1.2	1.9	1.1	2.4
	+	1.1	2.4	**3.0**	**5.4**
Dox	−	1.2	2.0	1.3	2.0
	+	1.0	2.0	**3.5**	**4.4**
Rif	−	4.2	3.4	4.8	3.8
	+	5.3	4.3	**8.0**	**5.0**
Chl	−	2.3	1.3	1.8	1.6
	+	2.0	1.5	**4.0**	**4.4**
Nor	−	0.1	0.2	0.1	0.3
	+	0.1	0.2	**0.5**	**0.9**
Nal	−	0.4	0.5	0.5	0.5
	+	0.4	0.6	**2.6**	**1.3**

[a] Strains were assayed by the E-test in the absence (−) or presence (+) of IPTG. MICs of *Y. pestis* bearing $marA47_{Yp}$ (on pRU1) in the presence of IPTG are shown in boldface. EV7651F/v, *Y. pestis* bearing pJPBH. Results are averages for four experiments. Antibiotic designations are as described for Table 2.

Ev7651F/RU1 also results in expression of $acrAB_{Yp}$, real-time PCR was performed (Materials and Methods). The levels of $marA47_{Yp}$ transcripts were 15-fold higher in the presence of 0.2 mM IPTG at both temperatures, while the $acrAB_{Yp}$ transcript was increased fourfold by 0.2 mM IPTG at both 26°C and 37°C. No difference was observed in the expression of other potential efflux pump genes, $acrEF_{Yp}$, and $emrAB_{Yp}$ in Ev7651F/RU1 transformants induced with 0.2 mM IPTG (data not shown). This result suggests that induction of multiple-drug resistance by $marA47_{Yp}$ in *Y. pestis* also functions via the AcrAB efflux pump.

***marA47*$_{Yp}$ expression is increased in spontaneous multiple-drug-resistant mutants of *Y. pestis*.** Five mutants chosen from independently selected early-log-phase cultures of EV7561F plated on BHI plates containing 5 μg/ml of tetracycline (see Materials and Methods) showed increased levels of resistance to multiple antibiotics. The mutants displayed differences in organic solvent tolerance (Table 4).

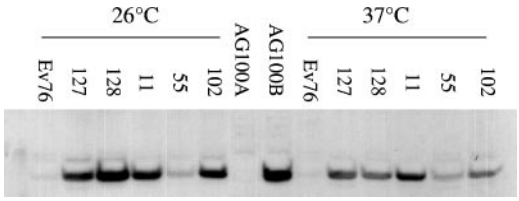

FIG. 2. AcrA expression in multidrug-resistant mutants of *Y. pestis*. AcrA expression was determined by Western blot assay using anti-AcrA antibodies (see Materials and Methods). *E. coli* AG100A deleted of *acrAB* and *E. coli* AG100B deleted of *acrR* (overexpressing AcrAB) served as negative and positive controls, respectively.

Real-time PCR demonstrated overexpression of $acrAB_{Yp}$ transcription in all five mutants. All except mutant 55 showed increased expression of $marA47_{Yp}$ at both 26°C and 37°C (Table 4). There was no direct correlation between the level of $marA47_{Yp}$ and $acrAB_{Yp}$ expression. No change was detected (by real-time PCR) in the levels of $acrEF_{Yp}$ and $emrAB_{Yp}$ expression for any of the mutants compared to those for parental strain EV7651F (data not shown). The enhanced $acrAB_{Yp}$ expression was confirmed by an increase in the AcrA protein seen in *Y. pestis* mutants by Western blot analysis using antibody to *E. coli* AcrA (Fig. 2). The relatively smaller increase in AcrA in mutant 55 was consistent with its relatively lower level of resistance.

The isolation of spontaneous multiple-drug resistance mutants of *Y. pestis* with associated increased expression of $marA47_{Yp}$ further supports the designation of $marA47_{Yp}$ as a functional ortholog of *marA* in *Yersinia*.

TABLE 4. Drug susceptibilities, organic solvent tolerances, and real-time PCR data for *Y. pestis* EV7651F spontaneous tetracycline-selected mutants at 26°C and 37°C (with 5mM $CaCl_2$)

Strain	MIC (μg/ml) of drug[a]												Growth with organic solvent[a]		Gene expression[b]			
	Tet		Dox		Rif		Chl		Nor		Nal		Hexane	Cyclo-hexane	$marA47_{Yp}$		$acrAB_{Yp}$	
	26°C	37°C	26°C	37°C	26°C	37°C	26°C	37°C	26°C	37°C	26°C	37°C	37°C	37°C	26°C	37°C	26°C	37°C
EV7651F (WT[c])	1.4	2.0	0.9	2.3	4.0	4.3	1.8	1.7	0.1	0.4	0.5	0.6	−	−				
Mutant 127	**3.7**	**3.3**	**8.0**	**6.0**	**6.0**	**6.0**	**8.0**	1.8	**0.4**	0.2	**3.7**	0.7	**+++**	**++**	++	++	+	+
Mutant 128	**5.3**	**3.7**	**8.0**	**4.7**	**8.3**	**6.7**	**12**	0.9	**0.5**	**0.8**	**5.0**	0.3	**+++**	**++**	++	++	+	+
Mutant 11	**3.0**	**6.0**	**7.3**	**13.3**	**5.3**	4.7	**9.3**	**5.3**	**0.3**	0.5	**1.7**	0.5	**+++**	**+++**	+++	++	+	+
Mutant 55	**2.7**	2.7	**4.0**	**6.0**	**6.0**	**5.3**	**3.7**	2.5	0.1	0.3	0.7	0.5	−	−	−	−	+	+
Mutant 102	**4.7**	**3.3**	**7.3**	**4.3**	**6.7**	**6.0**	**6.7**	2.0	**0.7**	0.2	**2.7**	0.6	**+++**	**+++**	++	+++	++	++

[a] For antibiotic designations and organic solvent descriptions, see Table 2. Results represent averages for three experiments. Reproducibly observed increased MICs compared to those for the wild type are presented in boldface. Mutants 127 and 128 appeared on day 2, mutant 11 on day 3, mutant 55 on day 4, and mutant 102 on day 5 of tetracycline selection.

[b] Expression was assayed by real-time PCR (see Materials and Methods). −, ≤1; +, 2- to 9-fold; ++, 10- to 50-fold; +++, >50-fold. No increase in expression was found for $acrEF_{Yp}$ or $emrAB_{Yp}$ at either temperature (data not shown).

[c] WT, wild type.

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