Characterization of the RND-Type Multidrug Efflux Pump MexAB-OprM of the Plant Pathogen *Pseudomonas syringae*[▽]

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In gram-negative bacteria, transporters belonging to the RND family are the transporters most relevant for resistance to antimicrobial compounds. In *Pseudomonas aeruginosa*, a clinically important pathogen, the RND-type pump MexAB-OprM has been recognized as one of the major multidrug efflux systems. Here, homologues of MexAB-OprM in the plant pathogens *Pseudomonas syringae* pv. phaseolicola 1448A, *P. syringae* pv. *syringae* B728a, and *P. syringae* pv. *tomato* DC3000 were identified, and *mexAB-oprM*-deficient mutants were generated. Determination of MICs revealed that mutation of MexAB-OprM dramatically reduced the tolerance to a broad range of antimicrobials. Moreover, the ability of the *mexAB-oprM*-deficient mutants to multiply in planta was reduced. RNA dot blot hybridization revealed growth-dependent regulation of the *mexAB-oprM* operon in *P. syringae*; the expression of this operon was maximal in early exponential phase and decreased gradually during further growth.

Bacteria have developed various ways to resist the toxic effects of antimicrobial compounds, such as enzymatic inactivation, alteration of the target structure, and reduced uptake (39). Another mechanism involves membrane-associated transport systems (23). Some transporters are specific and mediate the extrusion of a given drug or class of drugs. In contrast, so-called multidrug efflux (MDE) transporters can transport a wide range of structurally unrelated compounds.

The important role of efflux systems in multidrug resistance has been well documented for clinically relevant species. Some important bacterial pathogens have efflux mechanisms for all major classes of clinically available antibiotics (30, 38). However, the role of efflux pumps in clinically relevant bacterial pathogens is poorly understood in terms of physiological relevance. Genome sequence analysis revealed the highest numbers of MDE transporters in soil- or plant-associated bacteria (29). Plants are known to produce a wide range of structurally diverse secondary metabolites with antimicrobial activity (8, 40). Thus, it is tempting to speculate that MDE systems may confer resistance to plant antimicrobials and may contribute to the ability of the bacteria to colonize plants and cause disease. Consistent with this, inhibitors of MDE pumps have been used to increase the efficacy of plant antimicrobials up to 2,000-fold (36).

Only a limited number of transporters conferring multidrug resistance in phytopathogenic bacteria have been characterized. The RND-type transporter IfeAB has been identified in *Agrobacterium tumefaciens*. Palumbo et al. (28) showed that this transporter is able to export isoflavonoids, which are antimicrobial plant metabolites. The MFS-type efflux pump RmrAB in the nitrogen-fixing bean symbiont *Rhizobium etli* mediated

resistance to phytoalexins and was demonstrated to be involved in nodule formation (13). The *Xanthomonas oryzae* pigment xanthomonadin is transported by a putative RND-type transporter (12). The role of two RND-type and three MFS-type efflux systems in the pathogenesis of *Erwinia chrysanthemi* was analyzed by Maggiorani Valecillos et al. (24). Mutants with mutations in genes coding for these systems were differentially affected in terms of their virulence in different hosts and in terms of their susceptibility to antimicrobials.

Recently, two transporters belonging to the RND and MFS families were shown to be involved in secretion of phytotoxins and in self-protection. Kang and Gross (20) identified the RND-type PseABC system in the *sy-syp* genomic island of *P. syringae* pv. *syringae* required for biosynthesis of syringomycin and syringopeptin. Disruption of the *pseC* gene resulted in substantial reductions in phytotoxin secretion and in the virulence of the mutant. Bostock et al. (2) identified an MFS transporter, AlbF, involved in self-protection in an albicidin-producing *Xanthomonas albilineans* strain.

Previously, we demonstrated that the RND-type AcrAB efflux system plays an important role in virulence of *Erwinia amylovora*, the causal agent of fire blight in various members of the family Rosaceae, and that it is required for resistance to antimicrobial plant metabolites and for successful colonization of the host plant (4). Moreover, we identified NorM, a multidrug transporter belonging to the MATE family, which is involved in competition of *E. amylovora* with other bacteria. We demonstrated that NorM confers resistance to a toxin produced by *Pantoea agglomerans*, an epiphytic bacterium frequently found on apple flowers (5).

While the existence and role of RND-type pumps in many bacterial pathogens have been established, the occurrence and role of these efflux systems in MDE in *P. syringae* have not been conclusively demonstrated. *P. syringae* is an important agricultural pathogen that infects a tremendous diversity of important crop species. *P. syringae* strains are classified into approxi-

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mately 50 pathogenic varieties or pathovars based on the host plants from which they were originally isolated (17). In this study, we investigated the role of a putative MDE system, MexAB-OprM, in resistance to antimicrobials and in the virulence of three strains, *P. syringae* pv. phaseolicola 1448A (19), *P. syringae* pv. *syringae* B728a (10), and *P. syringae* pv. tomato DC3000 (3). The host ranges of these three strains are different; 1448A and B728a cause disease in beans, and DC3000 is pathogenic for tomato and *Arabidopsis*. The RND-type transporter of the *P. syringae* efflux pumps shared a high level of homology with MexB from *Pseudomonas aeruginosa*. In *P. aeruginosa*, MexAB-OprM has been recognized as one of the major MDE systems (30).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. syringae* strains were routinely maintained on mannitol-glutamate (MG) medium (21) at 28°C. For liquid cultures, bacteria were grown in HSC medium (27) or King's B (KB) medium (22). *Escherichia coli* strain DH5 α (33) cells were grown at 37°C on Luria-Bertani medium.

Drug susceptibility tests. The MICs of drugs for *P. syringae* strains were determined by a twofold dilution assay using Mueller-Hinton broth. All tests were done in triplicate by following the Clinical and Laboratory Standards Institute recommendations (7). Growth of bacteria at 28°C was examined by using visual inspection after 24 h of incubation.

Generation of Δ mexAB-oprM mutants. *P. syringae* unmarked Δ mexAB-oprM mutants were generated using the broad-host-range Flp-FRT recombination system (18). Two fragments, one located in the *mexA* gene and one located in the *oprM* gene, were PCR amplified using primer pairs mexR_fwd1 (5'-GCGTTGTCTGGCGCTGA-3')-mexA_rev1_KpnI (5'-TATGGTACCAGTCGCTGCCCACGGTGC-3') and oprM_fwd2_KpnI (5'-TATGGTACCAATGCCTACATGACCTGGC-3')-oprM_rev2 (5'-GGAGATGGTTTCGGTCTGC-3'). These primers were designed to bind at conserved sequences found in all three *P. syringae* strains. PCR products were cloned into pGEM-T Easy (Promega, Mannheim, Germany), yielding plasmids pGEM.mex and pGEM.opr. A 0.9-kb KpnI-SpeI fragment cut from pGEM.opr was ligated into KpnI-SpeI-digested pGEM.mex, yielding plasmid pGEM.mex-opr. A 1,825-bp KpnI FRT cassette fragment cut from pPS858-HW was ligated into KpnI-digested pGEM.mex-opr, yielding plasmid pGEM.mex-Gm-opr. The unique EcoRI site in the FRT cassette of pPS858 was eliminated by digesting the plasmid with EcoRI, blunt ending it, and rejoining it to form pPS858-HW. A 4.3-kb EcoRI fragment cut from pGEM.mex-Gm-opr was ligated into EcoRI-digested pEX18Ap, yielding the final replacement plasmid pEX18Ap.mex-Gm-opr. These plasmids were mobilized into *P. syringae* strains by triparental mating with helper plasmid pRK2013 (11). Putative mutants were screened for homologous recombination events by checking their antibiotic resistance (18). The FRT cassette was finally excised using plasmid pFLP2 (18). Mutants that were generated were verified by PCR using primers mexA_fwd (5'-GACATGCTCAAGCTGCGC-3') and oprM_rev (5'-CATCAGCCTGACGGCCAG-3'), which yielded an 800-bp fragment for the mutants.

Plant material and inoculation procedures. Bean plants (*Phaseolus vulgaris* cv. Red Kidney) were grown on shelves equipped with fluorescent lamps at 22 to 25°C with 60% humidity and with supplemental light using a 14-h photoperiod (350 microeinsteins m⁻² s⁻¹). *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia plants were grown in a controlled-environment chamber at 22°C with 80% humidity and a photoperiod with 8 h of light (170 microeinsteins m⁻² s⁻¹; cold white fluorescent light).

P. syringae strains, grown on MG agar for 24 h at 28°C, were suspended in distilled water, the concentration was adjusted to 1.0×10^7 CFU per ml, and the strains were applied to leaves of the host plants with an airbrush (~ 8 lb/in²) until the leaf surface was uniformly wet. Growth of bacterial strains was monitored by removing random leaf samples at 1 to 21 days postinoculation. Leaves were macerated in 10 ml isotonic NaCl per g (fresh weight). Bacterial counts (CFU per g [fresh weight]) were determined by plating dilutions of leaf homogenates onto MG agar.

EtBr efflux assay. Bacteria were grown on KB agar plates and suspended to an optical density at 600 nm of 0.1 in 100 mM NaCl-50 mM sodium phosphate buffer (pH 7.0) containing 0.05% glycerol (26). Fluorescence was measured at room temperature using a final volume of 1 ml. Ethidium bromide (EtBr) was added at a concentration of 1.6 μ g/ml (4 μ M), and carbonyl cyanide *m*-chloro-

phenylhydrazone (CCCP) was added at a concentration of 20 μ g/ml (100 μ M). Fluorescence was measured with an SFM25 spectrofluorometer (Kontron Instruments, Zürich, Switzerland).

RNA dot blot analysis. Total RNA was isolated by acid phenol-chloroform extraction (35). Aliquots of total RNA (200 ng per dot) were transferred to positively charged nylon membranes using the Minifold I spot blot system (Schleicher & Schuell BioScience, Dassel, Germany). The RNA hybridization probes were generated by in vitro transcription of PCR products. The *mexA*-specific primers mexA-RNA-fwd (5'-CTGCTCAGTGGCTGTAGC-3') and mexA-revT7 (5'-TAATACGACTCATATAGGAGGTGCGCAGCTTGAGCATGTC-3') were used to amplify PCR products from genomic DNA of 1448A, B728a, and DC3000. Digoxigenin-labeled RNA probes were synthesized by using digoxigenin-11-UTP and a Strip-EZ RNA probe synthesis and removal kit (Ambion Europe, Cambridgeshire, United Kingdom). Hybridization and signal quantification using an FLA-3000 phosphorimager (Raytest, Straubenhardt, Germany) were carried out by using our recently described method (35).

RESULTS AND DISCUSSION

Identification of the *mexAB-oprM* locus in *P. syringae*. A search with the BLASTP program of the National Center for Biotechnology Information using the amino acid sequence of MexB from *P. aeruginosa* PAO1 (accession number AAG03815) as the query identified homologous sequences in the genomes of *P. syringae* pv. phaseolicola 1448A (gene number PSPPH_4014), *P. syringae* pv. *syringae* B728a (gene number Psyr_4008), and *P. syringae* pv. tomato DC3000 (gene number PSPTO_4304). These homologues shared 79% amino acid sequence identity with MexB from *P. aeruginosa* PAO1 and 96% identity with each other. Upstream and downstream analyses of the sequences surrounding the *mexB* homologues revealed the associated genes (*mexA* and *oprM* homologues). We assumed that *mexA*, *mexB*, and *oprM* form an operon in *P. syringae*, based on the significant sequence homology and the genetic organization, which is similar to that of the *mexAB-oprM* genes in *P. aeruginosa* (31).

A gene encoding a transcriptional regulator was identified upstream of the *mexAB-oprM* structural genes, but in the opposite direction, in all three *P. syringae* genomes. The deduced amino acid sequences showed high degrees of identity to members of the TetR family of transcriptional repressors, including EmhR of *Pseudomonas fluorescens* (78% identity) (15), TtgR of *Pseudomonas putida* (70% identity) (37), and AcrR of *Erwinia amylovora* (46% identity) (4). However, no significant similarity was found to MexR, a member of the MarR family of bacterial transcriptional regulators, which negatively regulates the *mexAB-oprM* operon in *P. aeruginosa* (32). Therefore, we suggest renaming the PSPPH_4012, Psyr_4006, and PSPTO_4302 genes encoding a regulator of the *P. syringae* *mexAB-oprM* efflux systems *pmeR* (*Pseudomonas* multidrug efflux regulator). An intriguing question is why these bacteria use regulators belonging to different families to control the expression of their major MDE systems. The use of these regulators could reflect currently unknown physiological roles of the pumps in the adaptation of the bacteria to different environments.

Generation and genetic analysis of *mexAB-oprM*-deficient mutants. To study the influence of the MexAB-OprM efflux pump on antibiotic resistance and the virulence of *P. syringae*, *mexAB-oprM* deletion mutants were constructed. Unmarked mutants were generated by gene deletion using the Flp-FRT recombination system (18). Based on genome sequences, primers that amplify regions of the *mexA* and *oprM* genes were

designed and used for construction of gene replacement vectors, deleting a 3.9-kb fragment in the *mexAB-oprM* operon and affecting all three open reading frames present in this operon. Generated Δ *mexAB-oprM* mutants of 1448A, B728a, and DC3000 were verified by PCR analysis, which yielded 800-bp fragments which were absent from the parental strains. Sequencing of these PCR fragments revealed successful deletion of a 3.9-kb region in the *mexAB-oprM* operon and, as expected, the presence of a short *FRT*-containing sequence.

EtBr efflux/accumulation assay. An EtBr accumulation experiment was conducted to demonstrate that MexAB-OprM-mediated extrusion is a proton motive force-driven process. After addition of EtBr, a much lower level of fluorescence was observed in wild-type cells of all three *P. syringae* strains than in Δ *mexAB-oprM* cells, indicating that EtBr is a substrate for the MexAB-OprM efflux system (Fig. 1). Addition of CCCP to the assay mixture increased cellular EtBr levels in both types of cells. CCCP is an uncoupler of oxidative phosphorylation and breaks down the proton gradient on the membrane. The final levels of intracellular EtBr after addition of CCCP were similar in wild-type and mutant strains, indicating that the accumulated levels of EtBr in both strains were the same under deenergized conditions (Fig. 1). Importantly, the level of EtBr that accumulated in wild-type cells was much lower than the level in cells of the Δ *mexAB-oprM* mutant prior to addition of CCCP. This indicated that MexAB-OprM from *P. syringae* is a proton motive force-dependent EtBr efflux pump. However, addition of CCCP also increased intracellular EtBr accumulation in the Δ *mexAB-oprM* mutants, suggesting that additional proton motive force-dependent efflux systems are expressed in the absence of MexAB-OprM.

Substrate specificities of the MexAB-OprM efflux pump.

The susceptibilities of wild-type and Δ *mexAB-oprM* cells to a variety of antimicrobial agents were compared in an attempt to identify substrates of the MexAB-OprM pump. Deletion of MexAB-OprM resulted in increased susceptibility of all three *P. syringae* strains to almost all antimicrobials, plant-derived antimicrobials, detergents, and dyes tested (Table 1). Only the MICs of the aminoglycosides gentamicin, kanamycin, and streptomycin did not differ from those determined for the wild-type strains. The most striking result was the hypersusceptibility of the Δ *mexAB-oprM* mutants to the β -lactam antibiotics ampicillin, carbenicillin, and piperacillin. Comparison of the susceptibility profiles revealed that the MexAB-OprM efflux pumps of the three *P. syringae* strains showed the same substrate specificity. However, slight strain-specific variations in the level of resistance were found, which likely could be attributed to the activity of additional MDE pumps present in these strains. *P. syringae* pv. phaseolicola 1448A was generally more susceptible than the other two strains, especially to tetraphenylphosphonium chloride and dyes. Apart from this, strain 1448A showed high intrinsic resistance to fluoroquinolones. *P. syringae* pv. *syringae* B728a was more resistant to erythromycin than the other *P. syringae* strains. Moreover, strain B728a showed extremely high resistance to streptomycin. A BLAST search revealed the presence of Tn5393 in the genome of B728a (genes *Psyr_2667* to *Psyr_2670*). Tn5393 harbors the streptomycin resistance genes *strA* and *strB* encoding aminoglycoside phosphotransferases (6). This transposon is not present in strains 1448A and DC3000.

FIG. 1. Changes in EtBr fluorescence as measured using an excitation wavelength at 500 nm and an emission wavelength at 580 nm. EtBr (4 μ M) was added to the assay mixture to initiate the assay, and CCCP (final concentration, 100 μ M) was added at the time point indicated by the arrow. (A) *P. syringae* pv. phaseolicola 1448A (Δ) and 1448A. Δ *mexAB-oprM* ($\blacktriangledown$). (B) *P. syringae* pv. *syringae* B728a (Δ) and B728a. Δ *mexAB-oprM* ($\blacktriangledown$). (C) *P. syringae* pv. tomato DC3000 (Δ) and DC3000. Δ *mexAB-oprM* ($\blacktriangledown$). a.u., arbitrary units.

TABLE 1. Antimicrobial susceptibility profiles of *P. syringae* wild-type strains, $\Delta mexAB$ -*oprM* mutants, and complemented mutants

Antimicrobial	MIC (μ g/ml) for:								
	1448A	1448A. $\Delta mexAB$	1448A. $\Delta mexAB$ / pRK.mexAB	B728a	B728a. $\Delta mexAB$	B728a. $\Delta mexAB$ / pRK.mexAB	DC3000	DC3000. $\Delta mexAB$	DC3000. $\Delta mexAB$ / pRK.mexAB
Acridine orange	250	31	1,000	250	12.5	500	250	31	500
Acriflavin	7.8	0.3	15.6	31	5	62.5	31	2.5	62.5
Ampicillin	125	1.5	500	62.5	3.1	500	62.5	0.8	500
Benzalkonium chloride	25	3.1	50	25	6.2	25	25	6.2	50
Berberine	1,000	15.6	>1,000	1,000	62.5	>1,000	1,000	125	>1,000
Carbenicillin	1,000	6.2	>1,000	1,000	12.5	>1,000	500	3.1	>1,000
Cefoperazone	62.5	1.5	250	125	12.5	250	31	1.5	125
Chloramphenicol	25	1.5	250	50	6.2	250	31	1.5	125
Ciprofloxacin	1.5	0.1	10	0.3	0.01	0.3	0.06	0.01	0.3
Clindamycin	1,000	125	>1,000	1,000	125	>1,000	>1,000	250	>1,000
Crystal violet	25	0.8	25	25	3.1	25	25	1.5	50
Daunorubicin	100	6.2	>100	>100	25	>100	>100	25	>100
Erythromycin	25	3.1	125	100	25	125	12.5	3.1	31
Ethidium bromide	100	1.5	250	125	6.2	125	125	6.2	250
Fusaric acid	1,000	31	>1,000	1,000	62.5	1,000	1,000	62.5	>1,000
Fusidic acid	500	62.5	>1,000	1,000	125	>1,000	500	62.5	>1,000
Gentamicin	1.2	0.6	0.6	1.2	0.6	0.6	0.6	0.3	0.3
Kanamycin	1.2	0.6	1.2	2.5	2.5	1.2	0.6	1.2	0.6
Mitomycin C	3.1	0.1	10	2.5	0.2	5	1.2	0.1	5
Nalidixic acid	>1,000	31	>1,000	50	3.1	100	25	3.1	100
Naringenin	>1,000	250	>1,000	>1,000	500	>1,000	>1,000	250	>1,000
Nitrofurantoin	500	31	500	500	125	500	500	125	500
Norfloxacin	7.8	0.6	50	0.5	0.1	1.5	0.5	0.06	3.1
Novobiocin	>1,000	31	>1,000	1,000	31	>1,000	>1,000	31	>1,000
Phloretin	>1,000	250	>1,000	>1,000	500	>1,000	>1,000	250	>1,000
Piperacillin	31	0.6	62.5	62.5	3.1	125	15.6	0.3	31
Puromycin	125	3.1	1,000	500	31	1,000	125	15.6	1,000
Rhodamine 6G	125	1.5	500	250	15.6	250	500	31	1,000
Streptomycin	>100	>100	>100	3.1	3.1	3.1	6.2	3.1	3.1
Tetracycline	0.5	0.03		1	0.06		0.5	0.03	
Tetraphenylphosphonium chloride	125	1.2	500	1,000	125	1,000	1,000	31	>1,000
Trimethoprim	500	62.5	1,000	500	62.5	1,000	125	31	1,000

Heterologous complementation of $\Delta mexAB$ -*oprM* mutants.

Heterologous complementation of the $\Delta mexAB$ -*oprM* mutants in *trans* with plasmid pRK415.mexAB-*oprM*, which carried the 4.5-kb *mexAB*-*oprM* region from *P. aeruginosa*, restored resistance to all antimicrobials tested (Table 1). This suggested that the efflux pumps have similar substrate profiles. The levels of resistance of the *trans*-complemented mutants were higher than those of the wild-type strains. The complemented mutants were two- to threefold more resistant to most of the antimicrobials, suggesting that there was a gene copy effect due to the presence of the plasmid or differences in the expression levels due to different regulatory proteins and promoter regions, which share only 34 and 43% nucleotide sequence identity, respectively.

It is noteworthy that expression of MexAB-OprM from *P. aeruginosa* in the *P. syringae* mutants resulted in significantly higher levels of resistance to several antibiotics, such as chloramphenicol, ampicillin, and cefoperazone. This result suggested that despite similar substrate specificities, the MexAB-OprM efflux systems of *P. aeruginosa* and *P. syringae* have slight but significant differences in potency for extrusion of several substrates.

Transcriptional analysis of *mexAB*-*oprM* genes. To monitor *mexAB*-*oprM* transcript abundance in response to different growth media and growth phases, total RNA was prepared

from *P. syringae* cells grown in minimal medium (HSC medium) and complex medium (KB medium), respectively. Cells were harvested at distinct optical densities. The expression of the *mexAB*-*oprM* operon was examined by dot blot analysis with strain-specific 580-bp RNA probes directed to *mexA*.

Quantification of signals revealed that there was growth phase-dependent transcription of the *mexAB*-*oprM* operon in *P. syringae* (Fig. 2). The signal intensities for *mexA* mRNA in all three strains were highest during the early exponential growth phase and gradually decreased during further growth of the cultures. These results are in contrast to the results of reporter gene fusion experiments which showed that the expression of *mexAB*-*oprM* from *P. aeruginosa* is increased in stationary growth phase and enhanced by the quorum-sensing autoinducer *N*-butyryl-L-homoserine lactone (9, 25, 34). These contradictory findings indicate that the pumps may not have the same physiological function. Moreover, different methods were used to analyze *mexAB*-*oprM* expression. The proteins encoded by reporter genes are often very stable and may persist in the cells even after the corresponding promoter is shut down, thereby reflecting the overall activity of the promoter during growth.

Interestingly, a significant effect of the growth medium on *mexAB*-*oprM* expression was observed. The expression of the *mexAB*-*oprM* operon was higher in complex medium than in

FIG. 2. Bacterial growth and *mexAB-oprM* transcript abundance in *P. syringae* pv. phaseolicola 1448A, *P. syringae* pv. *syringae* B728a, and *P. syringae* pv. tomato DC3000. For liquid cultures, bacteria were grown in HSC medium and KB medium. (A) Growth kinetics of 1448A in HSC and KB media. (B) Quantitative RNA dot blot analysis for *mexAB-oprM* mRNA abundance in 1448A grown in HSC and KB media. (C) Growth kinetics of B728a in HSC and KB media. (D) Quantitative RNA dot blot analysis for *mexAB-oprM* mRNA abundance in B728a grown in HSC and KB media. (E) Growth kinetics of DC3000 in HSC and KB media. (F) Quantitative RNA dot blot analysis for *mexAB-oprM* mRNA abundance in DC3000 grown in HSC and KB media. The relative mRNA level was related to the highest mean value determined for a strain, which was defined as 100%. The data show the means and standard errors of two experiments with three replicates. OD_{600nm}, optical density at 600 nm.

minimal medium (Fig. 2). Moreover, the level of *mexAB-oprM* expression remained high until the late stationary growth phase in complex medium, whereas the level of *mexA* transcript signals decreased during later stages of bacterial growth in minimal medium. Either these data suggest that there is induction of *mexAB-oprM* expression by specific components of the complex medium or they may reflect a particular physiological function during growth in complex medium. The bacteria showed significantly increased growth rates in KB medium compared to the growth rates in the minimal HSC

medium; thus, MexAB-OprM might play a role in the secretion of metabolites important for fast-growing cells.

Contribution of MexAB-OprM to the virulence of *P. syringae*. The impact of MexAB-OprM on the virulence of *P. syringae* pv. phaseolicola 1448A, *P. syringae* pv. *syringae* B728a, and *P. syringae* pv. tomato DC3000 was evaluated by monitoring bacterial populations and by studying the development of disease symptoms in the host plants for 21 days (Fig. 3). The population size of *P. syringae* pv. phaseolicola 1448A reached a maximum of 1.3×10^9 CFU per g leaf tissue on bean, whereas

FIG. 3. Growth of *P. syringae* wild-type strains and Δ mexAB-oprM mutants in planta. (A) Growth of *P. syringae* pv. phaseolicola 1448A (▼) and 1448A. Δ mexAB-oprM (▽) on bean leaves. (B) Growth of *P. syringae* pv. syringae B728a (▼) and B728a. Δ mexAB-oprM (▽) on bean leaves. (C) Growth of *P. syringae* pv. tomato DC3000 (▼) and DC3000. Δ mexAB-oprM (▽) on leaves of *A. thaliana*. The symbols indicate the means for three independent leaf samples. The error bars indicate the standard deviations from the means ($n = 3$; $P < 0.005$).

the population size of the Δ mexAB-oprM mutant of this strain was 10-fold lower throughout the 21-day sampling period. The Δ mexAB-oprM mutant of *P. syringae* pv. syringae B728a showed an even greater reduction in virulence. The population size of strain B728a reached 1.4×10^7 CFU per g leaf tissue on bean, whereas the maximum population size of its Δ mexAB-

oprM mutant was 20-fold lower (1.3×10^5 CFU per g leaf tissue). The growth rates of *P. syringae* pv. tomato DC3000 and its Δ mexAB-oprM mutant were about the same until day 7. After day 7, the wild type continued to grow until the population size was 1×10^8 CFU/ml, whereas the population size of the Δ mexAB-oprM mutant was 10-fold lower. At day 21, the population sizes of both the wild type and the mutant were decreased, albeit to different degrees. The population size of the wild type (2.7×10^6 CFU per g leaf tissue) remained almost 200-fold greater than that of the mutant (1.4×10^4 CFU per g leaf tissue).

The disease symptoms caused by the Δ mexAB-oprM mutants were indistinguishable from those caused by the wild-type strains. However, consistent with the lower populations sizes, the symptoms caused by the Δ mexAB-oprM mutants were less abundant than the symptoms caused by the wild-type strains. Small, water-soaked spots developed on the undersides of the bean leaves inoculated with *P. syringae* pv. phaseolicola 1448A and its Δ mexAB-oprM mutant within 7 to 9 days after inoculation. These spots soon turned brown, and the tissues surrounding the spots became yellow. *P. syringae* pv. syringae B728a and B728a. Δ mexAB-oprM caused necrotic brown spots surrounded by narrow chlorotic halos that were visible within 9 to 12 days after inoculation. The disease symptoms caused by *P. syringae* pv. tomato DC3000 and DC3000. Δ mexAB-oprM consisted of small water-soaked lesions surrounded by large regions of chlorosis.

These results clearly indicated that disruption of MexAB-OprM resulted in reduced abilities of the bacteria to colonize their host plants. Plants synthesize a wide spectrum of secondary metabolites with antimicrobial activity (8, 14, 40), and it has been shown that MDE systems involved in resistance to such plant antimicrobials play a pivotal role in virulence (1, 4, 24). Thus, it is tempting to speculate that MexAB-OprM contributes to in planta growth of the bacteria by protecting them from the toxic effects of host antimicrobial compounds. Another possible explanation is based on the suggestion that MDE systems may play a role in the export of virulence factors. Hirakata et al. (16) demonstrated that the *P. aeruginosa* MexAB-OprM efflux system exports virulence determinants important for the bacteria to invade or translocate across epithelial cells. Whether MexAB-OprM in *P. syringae* is critical for the efflux of virulence factors in addition to its established role in exporting toxic substances remains to be elucidated.

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