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Genome-wide identification of genetic determinants of antimicrobial drug resistance in *Pseudomonas aeruginosa*

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ABSTRACT

The emergence of antimicrobial drug resistance is of enormous public concern due to the increased risk of delayed treatment of infections, increased length of hospital stay, substantial increase in cost of care and high risk of fatal outcome. A prerequisite for the development of effective therapy alternatives, is the detailed understanding of the diversity of bacterial mechanism that underlie drug resistance, especially in problematic gram-negative bacteria such as *Pseudomonas aeruginosa*. This pathogen provides impressive chromosomally-encoded mechanisms of intrinsic resistance, as well as the potential to mutate, gaining resistance to current antibiotics. In this study we have screened the comprehensive Harvard PA14 non-redundant library for *P. aeruginosa* mutants that either exhibited increased or decreased resistance against twenty antibiotics commonly used in the clinic. This approach identified several genes, whose inactivation sensitised the bacteria towards a broad spectrum of different antimicrobials and uncovered novel genetic determinants of resistance to various classes of antibiotics. Knowledge on the enhancement of bacterial susceptibility to existing antibiotics and on novel resistance markers or modifiers of resistance expression may lay the foundation to effective therapy alternatives and will be the basis for the development of new strategies in the control of problematic multi-resistant gram-negative bacteria.

INTRODUCTION

There is accumulating evidence that appropriate antibacterial therapy administered early in the course of an infection has major implications on the outcomes of severe bacterial diseases (20, 21, 43). Patients have greatly benefited from the introduction of effective antimicrobials in the last decades, however, the frequency and spectrum of antibiotic resistant infections have increased worldwide and substantial higher mortality rates were reported in patients given ineffective empiric therapy mainly due to resistance to the agents used (39). Today, in many intensive care units multi-drug resistant gram-negative bacteria pose the greatest therapeutic challenge. Therefore understanding the mechanisms of resistance and developing therapy alternatives for problematic gram-negative bacteria is of profound importance.

The increase in multi-drug resistance has been attributed to a combination of microbial characteristics, the selective pressure of antimicrobial use, and enhanced transmission of resistant organisms. This growing problem requires a comprehensive strategy that includes compliance with infection control principles, rational use of current antimicrobial agents, and development of new active agents. The diversity of bacterial mechanisms that underlie multi-drug resistance makes developing effective new antimicrobial agents very difficult, especially against problematic species such as *Pseudomonas aeruginosa*. This opportunistic gram-negative rod plays a dominant role as an infectious agent of the lungs of cystic fibrosis (CF) patients and has emerged as one of the most important human pathogens causing serious nosocomial infections. *P. aeruginosa* is capable to thrive in various environments and utilizes a broad spectrum of virulence factors to infect different hosts from plants and insects to humans. Due to intrinsic antibiotic resistance, *P. aeruginosa* infections are difficult to treat and are associated with high mortality rates (10). Intrinsic resistance to antibiotics seems to mainly result from the reduced permeability of the bacterial cell envelope and the activity of multi-drug efflux pumps, however, other yet to be discovered mechanisms might contribute.

Recent publications aimed at identifying the intrinsic "resistome" of *P. aeruginosa* by screening transposon mutant libraries for the resistance profiles towards several antibiotics (7, 16, 40, 45). From these studies it has become clear, that many previously unidentified genes play a role in antimicrobial resistance and that, depending on the antibiotic, there are obviously many modifiers of the expression of resistance in *P. aeruginosa*.

In this study we proceeded the identification of the *P. aeruginosa* resistome. We made use of a semi-automated antibiotic susceptibility test method and systematically screened the Harvard Medical School PA14 transposon mutant library (27) for mutants with either increased or decreased susceptibility towards 19 different antimicrobial agents commonly used in the clinic. This approach did not only uncover novel genetic determinants of resistance in *P. aeruginosa* towards specific antimicrobials compounds, but also identified genes whose inactivation sensitised the bacteria towards a broad spectrum of different antimicrobials. Our results provide valuable information for further studies aiming at predicting antibiotic resistance based on the genotype and on the other hand led to the identification of promising novel drug targets. Targets that enhance bacterial susceptibility could be the basis for the development of drugs that potentate existing antimicrobials and thus may function as chemosensitizers. Examples for the use of such novel antimicrobial combinations are the use of inhibitors of efflux pumps in combination with tetracycline in *E. coli* (30) or ciprofloxacin in *P. aeruginosa* (28).

METHODS

Bacterial strains

The PA14 transposon mutant library described by Liberati et al (27) was used to screen a comprehensive set of mutants for their resistance profile. This library was constructed using a mariner-based transposon containing the resistance cassette *aacC1* which confers resistance to

gentamicin but not tobramycin. The PAO1 transposon mutant library of the university of Washington (22) was used for verification and cross-referencing purposes. This library was constructed using two transposons derived from the IS50 element of the transposon Tn5, containing a tetracyclin resistance cassette.

Antimicrobial resistance screening

Semi-automated susceptibility testing with VITEK 2. PA14 mutants were streaked onto Columbia agar plates and incubated at 37°C overnight. A sufficient number of colonies was suspended in sterile saline (0.45 %) and adjusted to a 0.5 McFarland turbidity standard using the DensiChek Densitometer (bioMérieux). The inoculated tube was placed in a cassette on the VITEK 2 Smart Carrier Station. The sample number was entered and associated with an antimicrobial susceptibility test (AST) card. The Smart Carrier Station cassette with the cards and the test tubes was placed on the VITEK 2 instrument where the inoculation of the AST cards was automatically performed by the instrument.

Susceptibility testing by agar dilution. For standard agar dilution testing LB agar plates containing serial twofold dilutions of the antimicrobial agents to be tested were prepared, stored at 4°C and used within 5 days. Overnight grown *P. aeruginosa* mutants were diluted 1:100 in 0.9 % NaCl solution and replica plated onto the LB agar containing the respective antibiotics in twofold dilutions with duplicate plates for each single dilution. For testing both hyper-susceptible and hyper-resistant mutants, susceptibility was tested for single antibiotics belonging to six different classes: piperacillin (penicillins), meropenem (carbapenems), ceftazidime (cephalosporins), ciprofloxacin (fluoroquinolones), tobramycin (aminoglyocosides) and tetracycline (tetracyclines). The agar plates were incubated at 37°C and read at 18-24 h. The MIC was considered the lowest concentration of an antimicrobial agent that completely inhibited growth as detected visually.

Purification of membrane proteins

PA14 wild-type and the PA14 *oprF* transposon mutant were grown over night in 20 ml LB at 37 °C. Cells were harvested (6000 × *g*, 10 min) and resuspended in 100 mM CH_3COOK, 5 mM $(CH_3COO)_2Mg$ x 4 H_2O, 0.2 % (v/v) β-mercaptoethanol, 50 mM HEPES, pH 7.5, containing complete EDTA free protease inhibitor cocktail (Roche). Cells were lysed by French press (four times at 1,000 psi) and unbroken cells were removed by centrifugation. The supernatant was adjusted to 20 % sucrose and loaded onto a isopycnic sucrose gradient (3 ml of 80 % sucrose, 3 ml of 60 % sucrose, 13 ml 20 % sucrose/membrane fraction, 3 ml water cushion) and ultracentrifuged (100,000 × *g*, 1 h). Membranes enriched at the 20/60 % and 60/80 % sucrose interphase were collected from the gradient using a Pasteur pipette and washed by water in the presence of complete protease inhibitor cocktail (Roche). Associated proteins were rejected by carbonate extraction (17) and remaining membrane proteins were precipitated according to (44). 5 µg of both wild-type and mutant membrane proteins were suspended in loading buffer, separated on a 12 % SDS-polyacrylamide gel and stained by colloidal Coomassie blue.

RESULTS AND DISCUSSION

In this study we have screened an ordered comprehensive PA14 transposon mutant library (27) for mutants exhibiting either an increased or decreased susceptibility against various antimicrobials agents. Antimicrobial susceptibility testing (AST) was performed with the VITEK 2 System (bioMérieux, Marcy l'Étoile, France). This is an automated, short incubation micro-broth dilution system capable of performing susceptibility testing based on bacterial growth curves. 20 different antimicrobials agents were tested using the AST-N063 card at given concentration ranges. These included the two carbapenemes ertapenem (ETP) and meropenem (MEM), the two penicillins ampicillin (AMP) and piperacillin (PIP), both also in combination with beta-lactam inhibitors ampicillin/sulbactam (SAM) and

piperacillin/tazobactam (TZP), the seven cephalosporines (first - fourth generation) cefazolin (CFZ), cefoxitin (FOX), cefpodoxime (CPD), cefuroxime (CXM) cefotaxime (CTX), ceftazidime (CAZ), and cefepime (FEP), the aminoglycoside tobramycin (TOB), the three gyrase inhibitors ciprofloxacin (CIP), levofloxacin (LVX) and nalidixic acid (NAL), one tetracycline tigecycline (TGC), and trimetoprim/sulphmethoxazole (SXT). Table 1 summarizes the VITEK 2 results depicting the MIC values of 4676 single gene knock-out PA14 transposon mutants. Since the PA14 wild-type exhibited MIC values lower than the MIC calling range for PIP, TZP, MEM, FEP, CIP and TOB, only mutants that exhibited an increase in the resistance profile could be detected for these antibiotics (Table 1). Vice versa, the PA14 wild-type exhibited MIC values greater than the MIC calling range of AMP, SAM, ETP, CFZ, FOX, CPD, CXM, NAL and TGC. Thus, for these antibiotics only mutants that exhibited enhanced susceptibility could be detected. The MICs of CTX, CAZ, LVX, and SXT were within the given MIC calling range and therefore both mutants that exhibited an increased and a decreased susceptibility were identified. Additionally, the AST-N063 card includes the aminoglycoside gentamicin, which could not be used for the analysis because the transposon applied to create the PA14 mutant library carries a gentamicin resistance cassette (27).

Interestingly, while there was a significant overlap of mutants identified in this and previous screens (7, 40, 44) for the “intrinsic” resistome of the PA14 strain, only four mutants were identified in our screen and in the published screen on antibiotic resistant mutants in the Tb and 59.20 strain background (16). It is known that, while all *P. aeruginosa* strains share a large common set of “core genes”, their genetic repertoire exhibits a large variation in the “accessory genome” which has substantial influence on the phenotype of the strain. Although most of the mutations identified in the two studies affect common (core) genes of *P. aeruginosa*, the (accessory) genetic background might strongly affect the actual resistance phenotype.

Identification of hyper-susceptible *P. aeruginosa* mutants.

Using this global approach, we identified 40 PA14 mutants that exhibited an enhanced susceptibility against at least two out of 13 antibiotics or antibiotic combinations (Table 2). As the VITEK 2 system records MIC values on the basis of bacterial growth, we included the growth rate of these mutants in Table 2 (determined from the maximum slope of the growth curve of the AST control, growth rate of the wild type control was defined to be 1.0). Some mutants exhibited slight growth advantages whereas others grew slower and did not reach the maximal optical density (not shown) as compared to the wild-type. However, no mutant exhibited serious growth alterations.

We re-tested the susceptibility profile of these 40 mutants using the agar dilution method for six antimicrobials (PIP, MEM, CIP, tetracycline (TET), CAZ and TOB) and also confirmed the increased susceptible phenotype with the respective independent isolates from the PAO1 transposon mutant library (22). Table 2 lists the MIC changes for these mutants most of which could be confirmed to be hyper-susceptible towards several different antibiotics. Five of the 40 mutants could not be confirmed to be hyper-susceptible by the agar dilution method (PA3670, PA4456, PA4459, *fis* and PA5198).

Mutations within the *mexABoprM* multi-drug efflux pump, several outer membrane proteins and proteins involved in DNA replication, recombination and repair confer to hyper-sensitivity.

As expected, the two mutants harbouring a transposon insertion within the *mexA* and *mexB* genes, encoding for the major intrinsic multidrug *mexABoprM* efflux pump in *P. aeruginosa* (26, 35), exhibited the most pronounced overall susceptibility. They exhibited an enhanced sensitivity towards all classes of antibiotics tested in this screen. However, we also identified six mutants with insertions in genes involved in cell envelope biogenesis as being hyper-susceptible. These included mutants affected within *wzz*, encoding for a protein shown to be

important for determining the length of the O-antigen side chain attached to lipopolysaccharide (LPS) and to influence serum resistance (24), PA2800, encoding for a conserved hypothetical protein (putative lipoprotein) and genes encoding for the outer membrane proteins OstA, OprF, OmpH and BetT1. Outer-membrane proteins are key molecules with regard to the interface between the cell and its environment and these proteins have been suggested to influence the intrinsic resistance to antibiotics. Using a proteomic approach *P. aeruginosa* OmpH has been demonstrated to be an antibiotic-resistance-related protein (32) and a *Vibrio vulnificus ompH* mutant showed enhanced sensitivity to sodium dodecyl sulfate (SDS) and polymyxin B (2).

Ethanol tolerance in *Escherichia coli* appears to involve increased production of the *betT* encoded choline transporter (18), which also conveys general protection towards osmotic stress by mediating the uptake of choline, the precursor of the compatible solute glycine betaine (46). OstA has been suggested to contribute to n-hexane resistance by reducing the influx of n-hexane in *E. coli* (1). Furthermore disruption of the OstA protein in *Helicobacter pylori* resulted in altered membrane permeability, sensitivity towards organic solvent, and susceptibility to antibiotics (11).

OprF is the most common outer-membrane protein in *P. aeruginosa* and has been studied extensively. It is a non-specific porin permitting passive diffusion of small polar nutrients. Since the pore changes its channel size according to the growth conditions, this could affect outer-membrane permeability. OprF is also a promising candidate for a vaccine development, and is involved in maintaining cell shape and growth in a low-osmolarity environment (38, 48). Furthermore, it has recently been reported that the binding of human interferon-gamma to OprF activates quorum-sensing-dependent expression of virulence determinants (49). The role of OprF in antibiotic resistance remains controversial (6). It was suggested that loss of this protein may be involved in the multiple antibiotic resistance phenotype (33, 37) and it has been proposed that OprF has a role in antibiotic uptake in *P. aeruginosa* (9, 19, 47). As shown

in this study loss of OprF conferred to susceptibility against a very broad spectrum of antimicrobials such as SAM, ETP, CTX, CAZ, LVX, SXT and TGC whereas growth was not affected (Table 2). As depicted in Fig. 1 the OprF protein is completely absent from the outer membrane proteins confirming that the *oprF* transposon mutant in the PA14 strain background has lost expression of a functional OprF protein.

Within this global screen for hyper-sensitive *P. aeruginosa* mutants we also identified a *mucB* mutant exhibiting enhanced susceptibility towards CTX, CAZ, LVX and TGC. Inactivation of *mucB* causes conversion to mucoidy (29) and mucoid clinical isolates have been shown to tend towards more susceptible phenotypes (4).

In accordance with our findings, and despite the use of a different screening method, a recent study on the complex ciprofloxacin resitome has identified the PA14 outer membrane protein *betT1* and *oprF* transposon mutants as ciprofloxacin hyper-susceptible (7). Furthermore, the study revealed several mutants involved in DNA replication and repair to confer to increased ciprofloxacin sensitivity (*ruvA, recG, xerD, sss*) as well as *ftsK*, involved in cell division and chromosome partitioning. Our screen also revealed three genes encoding for proteins involved in DNA replication, recombination and repair (*topA, fis, ruvB*) to confer to broad spectrum hyper-sensitivity and we also identified the *ftsK* mutant exhibiting hyper-susceptibility towards ETP, CTX, CAZ and LVX. It has recently been suggested that bactericidal antibiotics induce cellular death by a common mechanism involving the generation of deleterious hydroxy radicals (25). It thus seems reasonable that the intrinsic "resistome" involves not only multi-drug efflux pumps and proteins that maintain the integrity of the cellular outer membrane, but also those that shield DNA from damaging agents and are involved in DNA repair processes. Proteins of similar functional categories have recently been identified to be of major importance for the *E. coli* and the *P. aeruginosa* intrinsic resistome in further studies (16, 42).

Identification of antibiotic resistant *P. aeruginosa* mutants.

We furthermore screened the library for mutants that exhibited an increase of the MIC for the 10 antibiotics, where resistance could be determined due to a MIC of the PA14 wild-type within or below the MIC calling range. 193 mutants showing increased resistance to at least one antibiotic are listed in Table 3.

Antibiotic resistance of 19 of those mutants were re-tested using the agar dilution method for (PIP, MEM, CIP, tetracycline (TET), CAZ and TOB) and we also confirmed the increased resistance profile with the respective independent isolates from the PAO1 transposon mutant library (Table 4).

As expected, in our VITEK 2 screen we were able to identify various genes which previously have been described to be involved in the alteration of the bacterial susceptibility profile. Among these genes were: *nfxB*, whose inactivation has been shown to lead to MexCD-OprJ-overproducing efflux mutants exhibiting resistance towards gyrase inhibitors and cefepime (36), *oprD* involved in carbapeneme resistance (34), *ampR,* encoding the transcriptional regulator of chromosomal AmpC beta-lactamase in *P. aeruginosa* (3), and *lpxC*. *lpxC* mutants exhibit increased resistance against CTX and LpxC-inhibitors have been demonstrated to show antibiotic properties. A recently discovered LpxC-inhibitor proved to control the growth of *E. coli* and *P. aeruginosa* with an efficacy rivalling that of CIP (5). We furthermore identified a *galU* mutant exhibiting increased cephalosporine and PIP resistance. A *galU* mutant was previously identified within a screen for aminoglycodide resistance (15), and it has been speculated that loss of the A- and B-band polysaccharides due to the impaired conversion of glucose-1-phosphate to UDP glucose (14) results in reduced antibacterial activity due to a lack of aminoglycoside binding to the bacterial cell (23). Interestingly, we also identified a *wbpM* mutant to exhibit increased resistance towards the cephalosporines and PIP. Insertional inactivation of *wbpM* was shown to result in mutants exhibiting three distinct

LPS phenotypes (14) suggesting that an altered LPS phenotype does not only affect susceptibility to aminoglycosides but also to beta-lactam antibiotics.

We furthermore identified *mutS* and *mutL* mutants which exhibited an increased resistance profile. Mutation within *mutS* and *mutL* has been described to lead to hypermutable phenotypes exhibiting a 100 – 1000 fold increased rate of spontaneous mutation (31). These mutants show enhanced acquisition of antibiotic resistance by accumulation of secondary mutations and are frequently found in chronic CF lung infections (12, 31). *RadA,* PA1767, PA5001 and *ahpF* mutants also exhibited antibiotic resistance in our screen; all of them have been picked in a previous screen of the PA14 mutant library for decreased susceptibility to different antibiotics (CIP, TOB and CAZ). Among those genes only *radA* was shown to be involved in the expression of an enhanced mutation frequency (45).

Aminoglycoside antibiotics must traverse the bacterial cytoplasmic membrane prior to initiating lethal effects, a process that seems to be dependent on the proton motive force (8, 13, 41). Thus resistance may be the result of a decreased aminoglycoside uptake. We found a mutant encoding for a protein involved in the production and maturation of the cytochrom c oxidase (PA1549) to exhibit enhanced resistance towards tobramycin. The respective PA14 transposon mutant has already been identified within a global screen for novel genetic determinants of aminoglycoside resistance (40). The reason for the significantly larger number of mutants identified in the screen by Schurek et al. (40) might be due to the fact that the PA14 wild-type MIC for tobramycin was below the calling range and therefore small MIC changes might have been missed.

Inactivation of the *nuoABDEFGHIJKLMN* operon encoding for the proton translocating type I NADH oxidoreductase did not result in an enhanced tobramycin resistance as observed by Schurek et al (40) however, we found an in enhanced resistance towards the cephalosporines (especially CTX) and PIP.

Concluding Remarks.

One approach to yield a new generation of useful antimicrobial compounds involves sensitisation of the bacteria to existing antibiotics by identifying targets for increasing susceptibility. Our finding that not only loss of efflux pumps but also loss of e.g. OprF entails pronounced hyper-susceptibility to various antibiotics belonging to different classes, enlightens OprF as a possible target candidate for the development of an attractive chemosensitizer.

We acknowledge that screening a transposon mutant library for mutants exhibiting an increased resistance profile is far from being comprehensive. Only non-essential genes can be identified and this screen will miss small mutations or a combination of several mutations that lead to the evolution of antibiotic resistant strains in the clinical setting. Nevertheless, screening of the Harvard PA14 transposon mutant library proved to be sufficient to detect many genes already predicted to play a role in *P. aeruginosa* resistance, and we identified a large number of previously unknown genes that modified the expression of resistance to many different antibiotics and which might turn out to be targets of clinical relevance. As the technology of high throughput sequencing and comparative genome hybridisation very rapidly evolves, it will be a very challenging task for the near future to identify novel genetic markers of antibiotic resistance and modifiers of resistance expression via genome wide association studies and to combine these data with the "resistome" data from our and other studies. The prediction of the antibiotic resistance profile of clinical strains based on their genotypes will be a major advancement for the rapid and reliable detection of antimicrobial resistance in modern microbiological diagnostics, and will significantly contribute to the efficient control of multi-resistance.

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Table 1. Antibiotic susceptibility testing of the Harvard *P. aeruginosa* PA14 transposon mutant library using the VITEK 2 system.

		beta lactams		beta lac. + b-lactamase inhibitor		carbape-nem beta-lactams		cephalosporins							(fluoro-) quinolones			amino-glyco-sides	other	
		AMP	PIP	SAM	TZP	ETP	MEM	CFZ	FEP	CTX	FOX	CPD	CAZ	CXM	CIP	LVX	NAL	TOB	TGC	SXT
calling range[a]	min	4	8	4/2	8/4	1	0.5	8	1	2	8	0.5	2	2	0.5	0.5	4	2	1	40
	max	16	64	16/8	64/4	4	8	32	32	32	32	4	32	32	2	4	16	8	4	160
MIC[b]																				
0.25							4639								4457	1775				
0.5						1	6								196	2864			6	
1						5	2		4500				93		1	8		4643	1	
2						13	2		134			4	3901		1	7		12	12	
4			4148		4156	126			13	2			639			2		3	23	
8			408	1	462	4502	1		2	260		4652	6						4612	8
16			47	4	6		5		2	2404			8				3			52
32		4654	5	4655	2					1854							4655			2869
64			6		20			4655	5	132	4654		5	4656						1723
128			43		10															2
failed[c]		22	19	16	20	29	21	21	20	24	22	20	24	20	21	20	18	18	22	22

[a]: minimum/maximum MIC (µg/mL) that can be determined with VITEK 2. Calling ranges are also indicated by a box in each column.

[b]: MIC distribution across all PA14 mutants is listed in one column per antibiotic. A MIC outside the calling range should be read as '≥' or '≤'. MIC of the PA14 wild type is marked by a gray shading. Please not that the MIC values for SXT are shown at the right margin of the table.

[c]: number of failed measurements.

Table 2. List of mutants hypersusceptible to at least two different antibiotics.

				$\log_2$ MIC change [a]							
PA14	**PAO1**	**name**	**growth** [b]	**SAM** [c,d]	**ETP** [c,d]	**CTX** [c]	**CAZ** [c]	**LVX** [c]	**NAL** [c,d]	**TGC** [c,d]	**SXT** [c]
PA14_05530	PA0425	*mexA*	1.07	-1 [f,g]	1 [f,g]	-1 [f,g]	≤ -1 [f,g]	≤ -1 [g]	-1 [g]	-2 [f]	≤ -2
PA14_05540	PA0426	*mexB*	1.03	-2 [f]	-4 [f]	-2 [f]	≤ -1 [f]	≤ -1 [g]	-1 [g]	[h]	≤ -2
PA14_07430	PA0572		1.05		-1 [f,g]	-1 [f]	≤ -1 [f]	≤ -1 [g]		[h]	-1
PA14_07770	PA0595	*ostA*	0.94	[h]		[h]	≤ -1 [f]				-1
PA14_54410	PA0764	*mucB*	1.35			-1 [f,g]	≤ -1 [f,g]	≤ -1 [g]		-1	
PA14_54330	PA0770[e]	*rnc*	0.78			[h]	≤ -1 [f]	≤ -1		-1 [f]	
PA14_53970	PA0794		1.19			-1 [f]	≤ -1 [f]	≤ -1 [g]			-1
PA14_53000	PA0871	*phhB*	0.66		-1 [g]	[h]	≤ -1 [f]			[h]	≤ -2
PA14_52260	PA0928		1.39			-1 [f]	≤ -1 [f]	≤ -1 [g]			-1
PA14_51780	PA0967	*ruvB*	1.15	[h]		-1 [f]	≤ -1 [f]	≤ -1			-1
PA14_51320	PA1005		1.01			[h]	≤ -1 [f]	≤ -1 [g]			-1
PA14_49320	PA1167		0.95		-1 [g]	-1 [f]	≤ -1 [f]	≤ -1 [g]		[h]	-1
PA14_47230	PA1316		1.01		-2 [g]	-1 [f]	≤ -1 [f]				-1
PA14_41570	PA1777	*oprF*	1.17	-1 [f,g]	-2 [f]	-1 [f,g]	≤ -1 [f,g]	≤ -1 [f,g]	[h]	≤ -4 [f]	≤ -2
PA14_40620	PA1848		1.39			-1 [f,g]	≤ -1 [f,g]	≤ -1 [g]			-1
PA14_38530	PA2008	*fahA*	0.77	-1	-1 [g]	-1 [f]	≤ -1 [f]			[h]	
PA14_30290	PA2615[e]	*ftsK*	1.31	[h]	-3 [f]	-2 [f]	≤ -1 [f]	≤ -1 [f]			
PA14_27920	PA2800		1.18			[h]	[h]	≤ -1 [g]		-2 [f]	-1
PA14_25110	PA3011	*topA*	0.88	[h]	-2 [f,g]	-1 [f,g]	≤ -1 [f,g]			≤ -4 [f]	
PA14_23890	PA3110		0.89		-2 [f,g]	-1 [f]	≤ -1 [f]				
PA14_23360	PA3160	*wzz*	1.12	[h]		[h]	≤ -1 [f]	≤ -1 [g]		-1 [f]	
PA14_22370	PA3233		1.07					≤ -1 [g]		-2 [f]	-1
PA14_20730	PA3351	*flgM*	1.00		-1	-1 [f,g]	≤ -1 [f,g]				-1
PA14_17170	PA3647[e]	*ompH*	1.07	[h]	-1 [f]	[h]	≤ -1 [f]	≤ -1 [f]	[h]	-2	-1
PA14_16890	PA3670		0.92		-2	-1	≤ -1	≤ -1 [g]			-1
PA14_14910	PA3800		1.07			-1 [f]	≤ -1 [f]	≤ -1 [f,g]	[h]	[h]	-1
PA14_12400	PA3976	*thiE*	1.12			-1 [f,g]	≤ -1 [f,g]	≤ -1 [g]			-1
PA14_12030	PA4005		0.85		-2 [g]	[h]	≤ -1 [f]			[h]	
PA14_08780	PA4269	*rpoC*	0.96	[h]		-1 [f,g]	≤ -1 [f,g]	≤ -1 [g]		-1 [f]	
PA14_57690	PA4441		1.00		-2 [g]	-1 [f]	≤ -1 [f]	≤ -1 [g]		[h]	-1
PA14_57880	PA4456		1.05			[h]	[h]	≤ -1 [g]		-1	-1
PA14_57910	PA4459[e]		1.31		-1	[h]	≤ -1 [f]	≤ -1		≤ -4	-1
PA14_62560	PA4727	*pcnB*	1.09			-1 [f]	≤ -1 [f]	≤ -1 [g]		-2	-1
PA14_62770	PA4745[e]	*nusA*	0.86		-3 [g]	-1 [f]	≤ -1 [f]	≤ -1 [f]	[h]	-1 [f]	-1
PA14_62880	PA4753		0.89	[h]	-2 [g]	[h]	≤ -1 [f]				
PA14_64190	PA4853[e]	*fis*	1.06			[h]	[h]	≤ -1		-1	-1
PA14_68670	PA5198		1.22		-2	-1	≤ -1	≤ -1		[h]	
PA14_69810	PA5288	*glnK*	0.71		-2 [g]	[h]	[h]			[h]	-1
PA14_70980	PA5375	*betT1*	1.23	[h]		-1 [f,g]	≤ -1 [f,g]	≤ -1 [f,g]	-1 [h]		-1
PA14_15750	- [e]		0.81		-1	-1 [f]	≤ -1 [f]	≤ -1		-2 [f]	

[a]: only those antibiotics for which a change in the MIC could be detected are lisited; only negative MIC changes are displayed.

[b]: maximum growth rate of the muntant growth control relative to wild type.

[c]: re-testing by agar dilution was performed using antibiotics of the same class: PIP for b-lactams (SAM), MEM for carbapenems (ETP), CAZ for cephalosporins (CAZ), CIP for

quinolones (LVX, NAL) and TET for tetracyclines (TGC), SXT was not retested by agar dilution.

[d]: MIC of PA14 wildtype was above the calling range (Table 1); MIC change calculated based on an assumed wildtype MIC of 2x the upper limit of the calling range.

[e]: no PAO1 mutant available.

[f]: MIC change consistent with agar dilution testing of the PA14 mutant using an antibiotic of the same class.

[g]: MIC change consistent with agar dilution testing of the PAO1 mutant using an antibiotic of the same class.

[h]: MIC was decreased in agar dilution testing of the PA14 mutant while the VITEK 2 screen predicted no change.

Table 3. List of mutants showing increased resistance

				log$_2$ MIC change [a]									
PA14	PAO1	name	growth [b]	PIP[c]	TZP[c]	MEM[c]	FEP[c]	CTX	CAZ	CIP[c]	LVX	TOB[c]	SXT
PA14_01080	PA0089		0.71					≥ 2					1
PA14_01100	PA0090		0.90	≥ 5	≥ 5		2	1	1				
PA14_01720	PA0140	*ahpF*	1.14	1	1		1	≥ 2	1				
PA14_02770	PA0227		1.00				2	1	1				1
PA14_03050	PA0247	*pobA*	0.97				2	*					
PA14_03760	PA0287		1.04	1	1		1	≥ 2	1		1		1
PA14_04110	PA0316	*serA*	1.18	1	1		1	≥ 2	1				
PA14_04430	PA0339		0.86	≥ 5	4		1		1				
PA14_04980	PA0381	*thiG*	0.74	≥ 5	4		1						
PA14_05050	PA0387		1.10	1	1		1	≥ 2	1				1
PA14_05310	PA0407	*gshB*	0.63						1	1	2		1
PA14_05410	PA0415		0.84	1	1			≥ 2	1				1
PA14_05620	PA0432	*sahH*	0.45	1	1			1		1	3		1
PA14_06010	PA0460		0.99	≥ 5	4		1	1					
PA14_06260	PA0479		1.40	1				≥ 2	1				
PA14_06950	PA0533		0.98	2	1		1	≥ 2	1				1
PA14_07070	PA0545		0.90	1	1			≥ 2	1				1
PA14_07780	PA0596		1.18	1	1		1	≥ 2	1				
PA14_07790	PA0597		1.15	2	1		1	≥ 2	1				
PA14_07950	PA0610	*prtN*	0.85	2				1					1
PA14_08520	PA0666		1.15	1	1		1	≥ 2	1				1
PA14_08540	PA0667		0.92	2	1		1	≥ 2	1	1			1
PA14_53820	PA0807		0.94	1	1	1		≥ 2	1				
PA14_53380	PA0842		1.03	1	1		1	≥ 2	1				1
PA14_52510	PA0908		1.02	2	1		1	≥ 2	1				1
PA14_52050	PA0944	*purN*	0.31				2	*	1	1			1
PA14_51880	PA0958	*oprD*	0.99			3							1
PA14_50980	PA1032	*pac*	0.82	≥ 5	1		1						
PA14_50250	PA1095		1.07	≥ 5	4		2	≥ 2	1				
PA14_50140	PA1101	*fliF*	0.98	1	1		2	≥ 2	1				
PA14_49320	PA1167		0.95	≥ 5									
PA14_49280	PA1171		0.75	≥ 5	2		1	1	1				
PA14_48840	PA1195		0.85	≥ 5	1		1	1	1				
PA14_47930	PA1259		0.83	≥ 5	4		1		1	1			
PA14_47300	PA1310	*phnW*	1.15	1	1		1	≥ 2	1		1		1
PA14_47230	PA1316		1.01	≥ 5									
PA14_46960	PA1338	*ggt*	1.07	1	1		1	≥ 2	1				1
PA14_46850	PA1347		0.99	1	1		1	≥ 2	1				
PA14_46840	PA1348		0.89	1	1	3	1	≥ 2	1		1		
PA14_45980	PA1428		1.07	1	1		1	≥ 2	1				1
PA14_45580	PA1459		0.65	1	1			≥ 2		1			*
PA14_44440	PA1549		0.85	2	2		2	≥ 2	2			1	
PA14_43950	PA1588	*sucC*	0.74	1	1		1	≥ 2	1				1
PA14_43920	PA1590	*braB*	1.03	1	1		1	≥ 2	1				1
PA14_43900	PA1592		0.88	4	1								
PA14_43670	PA1611		1.05	≥ 5	2		1						
PA14_43310	PA1639		0.85	1	1		1	≥ 2	1				1
PA14_43270	PA1643		1.11	1	1		1	≥ 2	1				
PA14_41710	PA1767		1.12	2	1	1	1	≥ 2	1				1
PA14_41530	PA1781	*nirB*	1.21	1	1		1	≥ 2	1				

Table 3 continued

PA14	PAO1	name	growth [b]	log$_2$ MIC change [a]									
				PIP[c]	TZP[c]	MEM[c]	FEP[c]	CTX	CAZ	CIP[c]	LVX	TOB[c]	SXT
PA14_40980	PA1821		1.12	≥ 5	4		1						
PA14_39420	PA1942		0.85	2	1			1	1				1
PA14_38350	PA2023	*galU*	1.16	≥ 5	≥ 5	1	3	≥ 2	2				
PA14_37550	PA2085		0.82	≥ 5	4		1	1	1				
PA14_37530	PA2086		1.23	1	1		1	≥ 2	1				
PA14_37310	PA2110		0.97	2						1			1
PA14_37030	PA2130	*cupA3*	1.26	1	1		1	≥ 2	1				
PA14_36280	PA2198		0.81	2									1
PA14_36170	PA2207		0.94	4	1								
PA14_34540	PA2326		0.93	≥ 5	1		2	1	1				
PA14_33720	PA2394	*pvdN*	1.05	≥ 5	1		1						
PA14_32860	PA2455		0.90	≥ 5	4		1	1					
PA14_32590	PA2478		1.11	1	1		1	≥ 2	1				1
PA14_32470	PA2487		1.14	1	1		1	≥ 2	1				
PA14_31850	PA2529		1.08	1	1		1	≥ 2	1				1
PA14_31820	PA2531		1.24	≥ 5	1								
PA14_31810	PA2532	*tpx*	1.11	2	1		2	≥ 2	1				
PA14_31400	PA2561		1.01	≥ 5	1		1						
PA14_30840	PA2571		1.01	≥ 5	4		1	1	1				
PA14_29970	PA2641	*nuoF*	0.91	2	1			1	1				
PA14_29930	PA2643	*nuoH*	0.96	2	1			≥ 2	1				
PA14_29920	PA2644	*nuoI*	1.03	2	1		1	≥ 2	1				
PA14_29900	PA2645	*nuoJ*	0.91	1	1			≥ 2	1				
PA14_29880	PA2647[b]	*nuoL*	0.90	2	1			≥ 2	1				
PA14_29880	PA2647[b]	*nuoL*	1.02	2	1			≥ 2	1	1			
PA14_29320	PA2691		1.14	1	1		1	≥ 2	1	1			
PA14_29290	PA2693		0.84	≥ 5	≥ 5	2	4	1	2				
PA14_29220	PA2700		1.10	1	1		1	≥ 2	1				1
PA14_27950	PA2797		0.77	1	1		1	≥ 2	1		1		1
PA14_26960	PA2871		1.11	1	1		1	≥ 2	1				
PA14_25880	PA2951	*etfA*	0.74	4	1			≥ 2	1				
PA14_25840	PA2953		1.05	2									
PA14_25820	PA2955		0.90	2	1			≥ 2	1				1
PA14_25630	PA2970	*rpmF*	0.97				2						
PA14_25420	PA2989		0.92	≥ 5	4		1	1	1				
PA14_25080	PA3014	*foaA*	0.95	1	1		1	≥ 2	1				1
PA14_25060	PA3015		0.87	2						1			
PA14_25050	PA3016		1.05	1	1		1	≥ 2	1				1
PA14_24690	PA3047		0.92	2	1		1	≥ 2	2				1
PA14_24490	PA3063	*pelB*	0.93	1	1			≥ 2	1				1
PA14_24150	PA3093		1.23	2	1		1	≥ 2	1				
PA14_23670	PA3127		1.01	2	1			1	1				1
PA14_23470	PA3141	*wbpM*	0.88	1	1		1	≥ 2	1				
PA14_23240	PA3170		0.93	≥ 5				1					1
PA14_23210	PA3172		0.90	2	1		1	≥ 2	1				1
PA14_22910	PA3194[b]	*edd*	0.46						1		2		
PA14_22910	PA3194[b]	*edd*	0.42			1					2		
PA14_22480	PA3224		1.01							2			
PA14_22330	PA3236		1.03	1				≥ 2	1				
PA14_21990	PA3247		0.93	1	1		1	≥ 2	1				1

Table 3 continued

PA14	PAO1	name	growth [b]	log$_2$ MIC change [a]									
				PIP[c]	TZP[c]	MEM[c]	FEP[c]	CTX	CAZ	CIP[c]	LVX	TOB[c]	SXT
PA14_21860	PA3259		1.26	1	1			≥ 2	1				
PA14_21410	PA3296	*phoA*	1.01					≥ 2					
PA14_21050	PA3324		0.83	≥ 5	4		1		1				
PA14_20730	PA3351	*flgM*	1.00	3									
PA14_19910	PA3416		1.24	1	1		1	≥ 2	1				
PA14_19340	PA3462		0.97	2				1					
PA14_19170	PA3472		0.87	≥ 5	1								
PA14_18080	PA3574		0.88	2	1	1	1	≥ 2	1				1
PA14_18060	PA3575		1.07	1	1		1	≥ 2	1				1
PA14_18050	PA3576		0.93	2	1		1	≥ 2	1				1
PA14_17500	PA3620	*mutS*	1.26	1	1			≥ 2	1				
PA14_16890	PA3670		0.92	≥ 5									
PA14_16500	PA3702	*wspR*	0.81	≥ 5	≥ 5		1	≥ 2	1				
PA14_16470	PA3704		1.05	1	1		1	≥ 2	1				
PA14_16280	PA3721		1.05	1	1		1	≥ 2	1				
PA14_16130	PA3733		1.13	1	1		1	≥ 2	1		1		1
PA14_14520	PA3826		0.90	1	1			≥ 2	1				1
PA14_14470	PA3831	*pepA*	0.67	2	1		1	≥ 2	1		2		1
PA14_14380	PA3837		0.93	≥ 5	3		1			1			
PA14_11900	PA4016		1.16	2	1		1	≥ 2	1				
PA14_11760	PA4025		0.92	2					1				1
PA14_10800	PA4109	*ampR*	1.08	3	3		1	≥ 2	3	1	1		1
PA14_09550	PA4204		1.09					≥ 2					
PA14_09480	PA4210	*phzA1*	1.21	1	1		1	≥ 2	1				
PA14_09300	PA4223		0.99							1			≥ 2
PA14_55770	PA4292		1.11		1			≥ 2	1				
PA14_56620	PA4354		1.09	1	1		1	≥ 2	1				1
PA14_56640	PA4355		1.13	1	1		1	≥ 2	1				
PA14_57080	PA4392		1.03					≥ 2					
PA14_57210	PA4402	*argJ*	1.00	1	1		1	≥ 2	3				1
PA14_57260	PA4406	*lpxC*	0.98					≥ 2					
PA14_57540	PA4429		0.87	2	2			≥ 2	2			1	
PA14_57560	PA4430		0.77	1	1			≥ 2					1
PA14_57570	PA4431		0.84	1	1			≥ 2	1			1	
PA14_57850	PA4454		1.06	≥ 5	4		1	1	1				
PA14_57950	PA4463		1.09	≥ 5	1			1	1				
PA14_58260	PA4490		0.85			2		1	1				1
PA14_58850	PA4536		0.83	1	1		1	≥ 2	1				
PA14_60860	PA4600	*nfxB*	0.79				1			3	3		1
PA14_60990	PA4609	*radA*	0.89	≥ 5									
PA14_62630	PA4733	*acsB*	1.02	1	1		1	≥ 2	1				1
PA14_63040	PA4766		0.91	2	1			1	1				
PA14_63210	PA4781		0.88	≥ 5	4		1						
PA14_63580	PA4811	*fdnH*	0.97	2						1			1
PA14_63970	PA4838		1.07	2	1		1	≥ 2	1				1
PA14_65250	PA4939		1.11	1	1		1	≥ 2	1				1
PA14_65280	PA4942	*hflK*	1.05	1	1		1	≥ 2	1				1
PA14_65320	PA4945	*miaA*	0.65	2	2		1	1	1	1			1
PA14_65350	PA4946[b]	*mutL*	1.30	2	1		1	≥ 2	1				1
PA14_65350	PA4946[b]	*mutL*	1.12	1	1		1	≥ 2	1				

Table 3 continued

				log$_2$ MIC change [a]									
PA14	PAO1	name	growth [b]	PIP[c]	TZP[c]	MEM[c]	FEP[c]	CTX	CAZ	CIP[c]	LVX	TOB[c]	SXT
PA14_65750	PA4974		1.01	2									
PA14_66120	PA5001		0.89	2	1		1	≥ 2	3				1
PA14_66150	PA5003		1.11	2	1		1	≥ 2	2				1
PA14_66480	PA5028		1.16	1	1		1	≥ 2	1				1
PA14_66580	PA5037		0.96	2				1	1				
PA14_66600	PA5038	*aroB*	0.55	4	4		3	≥ 2	3	1	2		
PA14_67270	PA5094		0.99										≥ 2
PA14_67530	PA5114		1.09	≥ 5	4		1		1				
PA14_67670	PA5124	*ntrB*	0.93	≥ 5	4		1						
PA14_68580	PA5192	*pckA*	1.05	2	1		1	1	1				
PA14_68610	PA5193	*hslO*	0.78	≥ 5	≥ 5		1	1					
PA14_68680	PA5199	*envZ*	1.06	1	1		1	≥ 2	1				1
PA14_68730	PA5203	*gshA*	0.59								2		
PA14_68800	PA5208		0.91	1	1		1	≥ 2	1				1
PA14_69000	PA5224	*pepP*	0.72	2	1		2	1	1				1
PA14_69170	PA5238		0.89	1	1			≥ 2					
PA14_69270	PA5246		0.87	≥ 5	1								
PA14_70860	PA5369		0.84	1	1			≥ 2	1				1
PA14_71630	PA5427	*adhA*	1.10	1	1		1	≥ 2	1				
PA14_71700	PA5433		1.09	1	1		1	≥ 2	1				1
PA14_72140	PA5466		1.11	1	1		1	≥ 2	1				1
PA14_73200	PA5551		1.19	1	1		1	≥ 2	1				
PA14_73370	PA5565[b]	*gidA*	0.62								2		
PA14_73370	PA5565[b]	*gidA*	0.78	1	1		2	1	1				
PA14_73410	PA5568		0.81	2	1			1	1				1
PA14_14420	-		1.03	1	1		1	≥ 2	1				1
PA14_15540	-		1.13	1	1		1	≥ 2	1				1
PA14_15600	-		0.93	1	1		1	≥ 2	1				1
PA14_18070	-		0.92	2	2	1	2	≥ 2	1				1
PA14_23420	-		0.83	2	1		1	≥ 2	1				1
PA14_23430	-		1.21	1	1		1	≥ 2	1				1
PA14_23460	-	*orfN*	0.92	2	1		1	≥ 2	1				1
PA14_28520	-		0.91	1	1		1	≥ 2	1				1
PA14_43090	-		0.91	1	1		1	2	1				1
PA14_44230	-		1.19	1	1		1	≥ 2	1				
PA14_51950	-		1.04	1	1		1	≥ 2	1				1
PA14_54750	-		1.17	1	1		1	≥ 2	1				
PA14_58760	-	*pilC*	0.73	2	1		1	≥ 2	1				1
PA14_66100	-		1.08	1	1		1	≥ 2	1				1
PA14_30210	-[b]	*clpS*	1.20	3	4		1	≥ 2	3				1
PA14_30210	-[b]	*clpS*	1.18	3	1		1	≥ 2	1				1
PA14_60280	-[b]	*fimU*	0.82	2	1		1	≥ 2	1		1		
PA14_60280	-[b]	*fimU*	0.80	1	1		1	≥ 2	1				

[a]: only those mutants are listed that exhibted at least a 4 fold increase in MI; only positive MIC changes are displayed.

[b]: independent strains with transposon in different positions within the gene.

[c]: MIC of PA14 wildtype was below the calling range (Table 1); MIC change calculated based on an assumed wildtype MIC of 0.5x the lower limit of the calling range.

*: failed measurement.

Table 4. Re-testing of mutants exhibiting increased resistance

			log_2 MIC change[a]				
PA14	**PAO1**	**gene name**	**PIP**[b]	**MEM**[b]	**CAZ**	**CIP**[b]	**TOB**[b]
PA14_01100	PA0090		≥ 5 [d,e]		1		[f]
PA14_51880	PA0958	*oprD*		3 [d]			
PA14_50250	PA1095		≥ 5 [d,e]	[f]	1 [d]	[f]	[f]
PA14_46840	PA1348		1 [d,e]	3 [d]	1		
PA14_44440	PA1549		2 [d,e]		2 [d]		1 [d,e]
PA14_38350	PA2023	*galU*	≥ 5 [d,e]	1 [d]	2 [d]		[f]
PA14_29290	PA2693		≥ 5 [d,e]	2	2		
PA14_22480	PA3224					2 [d,e]	
PA14_16500	PA3702	*wspR*	≥ 5 [d,e]	[f]	1 [d]		[f]
PA14_14470	PA3831	*pepA*	2 [d,e]	[f]	1 [d,e]		
PA14_10800	PA4109	*ampR*	3 [d,e]	[f]	3 [d]	1 [d]	[f]
PA14_57540	PA4429[c]		2		2		1 [d]
PA14_57570	PA4431		1 [d,e]		1 [d]		1 [d]
PA14_58260	PA4490			2 [d,e]	1 [e]		
PA14_60860	PA4600	*nfxB*	[f]			3 [d,e]	
PA14_66600	PA5038	*aroB*	4 [d,e]	[f]	3 [d]	1 [d]	
PA14_68610	PA5193	*hslO*	≥ 5 [d,e]	[f]	[f]		[f]
PA14_18070	- [c]		2	1 [d]	1 [d]	[f]	
PA14_30210	- [c]	*clpS*	3 [d,e]		3 [d]	[f]	[f]

[a]: only positive MIC changes are displayed.

[b]: MIC of PA14 wildtype was below the calling range (Table 1); MIC change calculated based on an assumed wildtype MIC of 0.5x the lower limit of the calling range.

[c]: no PAO1 mutant available.

[d]: MIC change consistent with agar dilution testing of the PA14 mutant with the same antibiotic.

[e]: MIC change consistent with agar dilution testing of the PAO1 mutant with the same antibiotic.

[f]: MIC was increased in agar dilution testing of the PA14 mutant while the VITEK 2 screen predicted no change.

FIGURE LEGENDS.

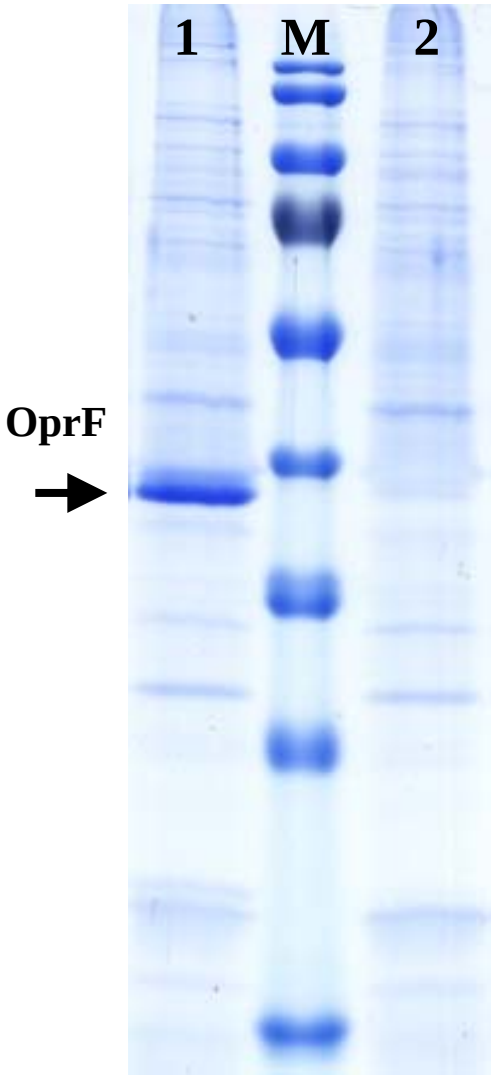

Figure 1. SDS-PAGE of membrane proteins. As opposed to the PA14 wild-type (lane 1) the PA14 *oprF* mutant (lane 2) is missing the OprF band at 37.6 kDa (arrow). M – Prestained PageRuler™ protein ladder (Fermentas).

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