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Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation

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Colonization of the lungs of cystic fibrosis (CF) patients by the opportunistic bacterial pathogen *Pseudomonas aeruginosa* is the principal cause of mortality in CF populations[1,2]. *Pseudomonas aeruginosa* infections generally persist despite the use of long-term antibiotic therapy[1,3]. This has been explained by postulating that *P. aeruginosa* forms an antibiotic-resistant biofilm[4,5] consisting of bacterial communities embedded in an exopolysaccharide matrix. Alternatively, it has been proposed that resistant *P. aeruginosa* variants may be selected in the CF respiratory tract by antimicrobial therapy itself[1,6]. Here we report that both explanations are correct, and are interrelated. We found that antibiotic-resistant phenotypic variants of *P. aeruginosa* with enhanced ability to form biofilms arise at high frequency both *in vitro* and in the lungs of CF patients. We also identified a regulatory protein (PvrR) that controls the conversion between antibiotic-resistant and antibiotic-susceptible forms. Compounds that affect PvrR function could have an important role in the treatment of CF infections.

Antibiotic-resistant colonies of *P. aeruginosa* clinical isolate PA14 (ref. 7) arose at a frequency of 10^{-6} to 10^{-7} when cultures were plated on Luria-Bertani (LB) agar containing kanamycin (200 $\mu g\,ml^{-1}$). Resistant colonies were smaller than wild-type even on antibiotic-free media, and exhibited colony morphologies similar to the ones described for CF variant isolates[6,8]. One class of the resistant variants (approximately 30%) exhibited a rough colony phenotype compared with the wild type, and was called RSCV (rough small-colony variant). When RSCVs were grown on antibiotic-free LB agar, wild-type revertants, characterized by a large colony size, smooth appearance, and wild-type levels of susceptibility to kanamycin, arose on the edges of the variant colonies after five days incubation at room temperature (Fig. 1a), suggesting that the phenotypic changes observed in the resistant variants were transient. In addition to being resistant to kanamycin, (40 times the wild-type susceptibility level), individual RSCV colonies were also resistant to amikacin (30 $\mu g\,ml^{-1}$), carbenicillin (300 $\mu g\,ml^{-1}$), gentamicin (30 $\mu g\,ml^{-1}$), tobramycin (10 $\mu g\,ml^{-1}$) and tetracycline (150 $\mu g\,ml^{-1}$). Consistent with this latter result, resistant variants were also obtained at frequencies of about 10^{-7} by plating cultures of PA14 on media containing similar concentrations of the antibiotics mentioned above.

Although RSCV colonies were smaller than wild type, their small colony size was not a consequence of slow growth, as the generation time of RSCV in liquid medium was not significantly different from that of the wild type, even in LB agar containing 200 $\mu g\,ml^{-1}$ kanamycin. Unlike the wild type, RSCV formed visible aggregates when liquid cultures were left without shaking at room temperature (Fig. 1b). Moreover, RSCVs exhibited increased attachment to glass (not shown) and polyvinylchloride plastic (PVC) (Fig. 1c). Reverted RSCV showed wild-type levels of both agglutination and attachment to glass and PVC plastic (data not shown).

Because the ability of bacteria to attach to each other and to surfaces depends in part on the interaction of hydrophobic domains[9], we determined the surface hydrophobicity of RSCV relative to the wild type. RSCV clones agglutinated at a lower salt concentration (0.125 M) than wild-type PA14 (0.5 M), indicating that RSCVs had a higher degree of surface hydrophobicity. Similarly, 200 mM tetramethyl urea (TMU), a hydrophobic bond-breaking agent, reduced the attachment of RSCV cells to PVC plates to wild-type levels (data not shown). Several RSCV clones were tested in the experiments described above, and all exhibited similar phenotypes. A single RSCV clone, referred to as RSCV for simplicity, was therefore chosen for further analysis.

Recently, it has been shown that *P. aeruginosa* in the sputum of CF patients exists primarily as a biofilm[4]. To determine whether the antibiotic-resistant phenotype of RSCV is associated with altered biofilm formation, biofilms of PA14 or RSCV expressing green fluorescent protein (GFP) were cultivated in flow chambers under continuous culture conditions. Analysis of biofilm structures using confocal scanning laser microscopy (CSLM) showed that RSCV formed biofilm faster (RSCV microcolonies appeared 4–5 h earlier

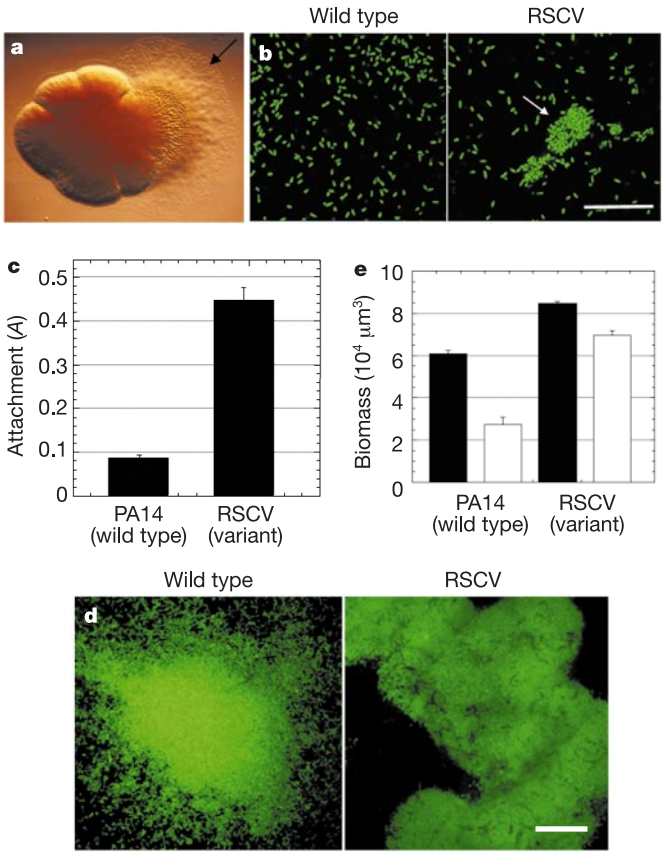

Figure 1 Characterization of *P. aeruginosa* antibiotic-resistant RSCV. **a**, Reversion of RSCV was observed on the edges of colonies (arrow) on antibiotic-free media, 30 × . **b**, CSLM analysis of bacterial aggregates (arrows) expressing GFP after growth in liquid broth. Scale bar, 25 μm. **c**, Attachment of wild-type PA14 and RSCV to PVC (see Methods). *A*, absorbance. **d**, CSLM analysis of biofilm formed by wild-type PA14 and RSCV expressing GFP. Scale bar, 50 μm. **e**, Resistance of biofilms to tobramycin was determined by measuring viable biomass on 45-h-old established biofilms before (filled bars) and after (open bars) 36-h tobramycin (200 $\mu g\,ml^{-1}$) treatment. In the absence of antibiotic treatment, both PA14 and RSCV biofilms grew at constant rates (not shown).

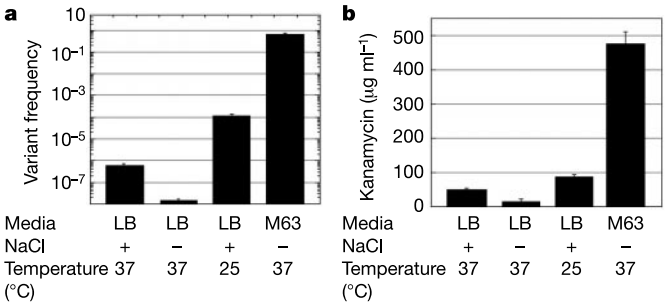

Figure 2 Appearance of phenotypic variants resistant to kanamycin depends on environmental factors. **a**, Effect of different environmental stimuli on the rate of appearance of antibiotic-resistant variants on different media containing 200 μg ml^{-1} kanamycin (see Methods). **b**, Minimal inhibitory concentrations of kanamycin for strain PA14 using different conditions (MICs were determined as described previously[25]).

than wild type) and had more biomass than wild type (Fig. 1d). Moreover, wild type and RSCV displayed different patterns of biofilm development. After 21 h, PA14 wild-type formed flat circular microcolonies, whereas RSCV formed irregularly shaped three-dimensional structures that did not show the typical *P. aeruginosa* biofilm morphology (Fig. 1d). Additionally, measurements of viable biomass of GFP-tagged PA14 and RSCV cells using CSLM analysis[10] showed that biofilms formed by RSCV were more resistant to a continuous flow of tobramycin (200 μg ml^{-1}) than wild-type PA14 biofilms (Fig. 1e), paralleling the resistance observed on plates.

Phenotypic (phase) variation is a common phenomenon in Gram-negative bacteria that often involves environmentally regulated changes in surface components leading to alterations in observable phenotypes[11]. We therefore examined the effect that different environmental stimuli had on the appearance of kanamycin-resistant phenotypic variants. As shown in Fig. 2a, we observed a 40-fold increase in the frequency of appearance of resistant variants (not just RSCV) obtained on LB media containing NaCl (85 mM) compared with the same medium without NaCl. Moreover, the frequency of variants increased 200-fold when plates were incubated at 25 °C compared with 37 °C (Fig. 2a). Finally, a marked 10^6-fold increase was obtained on minimal M63 salts compared with LB medium (Fig. 2a). Notably, there was a correlation between the frequency of appearance of kanamycin-resistant variants on plates and minimal inhibitory concentrations (MICs) of kanamycin in liquid culture (Fig. 2b). For example, the high frequency of resistant variants obtained on M63 plates correlated with the relatively high concentration of kanaymycin (475 μg ml^{-1}) required to inhibit the growth of PA14 in M63 liquid medium (Fig. 2a, b). These data suggest that the components involved in the formation of antibiotic-resistant variants are regulated by environmental signals. Moreover, the data indicate that the portion of the population that becomes resistant to antibiotics through phenotypic variation is dependent on environmental conditions.

To investigate whether antibiotic treatment in *P. aeruginosa* CF infections selects for resistant variants, we looked for the presence of small-colony variants in CF sputum samples. The analysis of five CF sputum samples obtained from the Clinical Microbiology Laboratory at Massachusetts General Hospital revealed that two of the samples (5 and 38) contained 100% small-colony variants (Table 1) that reverted to wild type on prolonged incubation on antibiotic-free medium. Importantly, both samples 5 and 38 corresponded to patients that were undergoing antibiotic treatment at the time the samples were obtained (intravenous amikacin/ceftazidime for two days, and oral levofloxacin/inhaled tobramycin for six weeks, respectively; Table 1). Moreover, there was 29% enrichment in small-colony variants in samples taken on two consecutive days from the patient that was undergoing intravenous antibiotic treatment. As shown in Table 1, 30–100% of the small-colony variants present in samples 5 and 38 were resistant to four different antibiotics (amikacin, gentamicin, tetracycline and tobramycin) at concentrations equal to or higher than the minimal bactericidal concentration (MBC) of their respective reverted colonies. Although the three other CF sputum samples (41, 42 and 43) appeared to contain either a small proportion or no detectable small-colony variants when plated on antibiotic-free media, they did contain a considerable number (0.5–15%) of antibiotic-resistant variants (Table 1). This discrepancy was due to the fact that it took the small-colony variants 36–40 h to form visible colonies, at which time the fast-growing wild-type bacteria present in the sputum samples had overgrown the antibiotic-free plates.

Previous reports have shown that phenotypic switching in *Pseudomonas tolaasii* is modulated by an environmentally responsive regulatory factor[12]. To identify putative *P. aeruginosa* factor(s) involved in the switching between antibiotic resistant and susceptible forms, we transferred a cosmid library of PA14 chromosomal DNA[7] en masse to RSCV, and screened 2,500 transconjugants for colonies displaying wild-type colony size and morphology on antibiotic-free LB medium. We identified a cosmid clone (pED20) that was capable of greatly enhancing the rate of RSCV reversion. Whereas RSCV colonies were normally very stable in liquid culture (that is, no revertants were observed after 12 h incubation of an overnight culture plated on LB agar), 100% of the pED20-carrying RSCV cells formed wild-type colonies on LB plates after overnight incubation. A subclone of pED20, pED202, which contained a 3.5-

Table 1 Occurrence of phenotypic variation in *P. aeruginosa* CF isolates

	Sputum samples				
	Sample 5	Sample 38	Sample 41	Sample 42	Sample 43
Antibiotic treatment of CF patients	Amikacin (IV), ceftazidime (IV)	Tobramycin (I), levofloxacin (O)	None	None	None
Small-colony variants in sample (%)	100	100	<0.11	0	<0.12
Variants resistant to amikacin (%)	100	100	15	5	0.2
Variants resistant to gentamicin (%)	100	100	10	6.6	0.5
Variants resistant to tetracycline (%)	30	32	0	0	ND
Variants resistant to tobramycin (%)	50	100	0.10	0	0.5

The modes of antibiotic administration in CF patients were: intravenous (IV), inhalation (I), or oral (O). ND, not determined.

kilobase (kb) fragment, restored the attachment (Fig. 3a), colony morphology and auto-agglutination phenotypes (data not shown) of RSCV cells to wild-type.

The pED202 clone contains a single open reading frame (ORF) that shows sequence similarities to response regulator elements of two-component regulatory systems, including 30% identity and 45% similarity to the *Vibrio cholerae* response regulator VieA. The ORF on pED202 (designated *pvrR* for phenotype variant regulator) also shows 29% identity and 45% similarity to a probable two-component response regulator identified in *P. aeruginosa* strain PAO1 (ref. 13) (ORF PA3947). A homology search against domain sequences (ProDom; http://prodes.toulouse.inra.fr/prodom/doc/prodom.html) identified four regions with high-scoring segment pairs in PvrR (Fig. 3b). All four domains are also present in VieA and the PAO1 putative regulator (Fig. 3b). Moreover, these four domains exhibit high levels of amino-acid sequence similarity (30–60%; Fig. 3b). Sequence analysis of the regions located upstream and downstream of *pvrR* revealed the presence of two additional ORFs (designated ORF1 and ORF3; Fig. 3c) with sequence homology to two-component regulatory elements. The protein coded for by ORF1 has homology to probable sensor/response regulator hybrids from *P. aeruginosa* (33% identity and 46% similarity to ORF PA2824), and the protein coded for by ORF3 shows 46% identity and 63% similarity to the GacS sensor kinase from *P. fluorescens*. To determine whether *pvrR* or a highly similar *pvrR* homologue is present in other *P. aeruginosa* strains, we performed polymerase chain reaction (PCR) analysis of 14 *P. aeruginosa* strains using PvrR-specific primers. We subsequently confirmed the specificity of the PCR products obtained by Southern blotting and hybridization with a *pvrR*-specific probe. Seven out of seven CF isolates, two out of three clinical isolates and three out of four standard *P. aeruginosa* laboratory strains contained the *pvrR* gene fragment or a highly similar fragment (data not shown).

Consistent with the putative role of PvrR in the regulation of phenotypic switching, overexpression of PvrR from the pED202 clone in wild-type PA14 resulted in a sixfold reduction in the frequency of resistant variants obtained after plating overnight cultures on kanamycin plates (200 $\mu g\,ml^{-1}$) compared with wild-type (Fig. 3d). Interestingly, the PvrR-overexpressing strain also caused a 2.5-fold reduction in attachment to PVC plastic with respect to the strain carrying the vector alone (Fig. 3e).

As PvrR is involved in the regulation of the phenotypic switch, we postulated that mutation of *pvrR* would alter the proportion of resistant variants present in the PA14 population. Indeed, a 914-bp in-frame deletion of *pvrR* (*ΔpvrR)* in PA14 exhibited increased frequency of appearance of resistant variants on kanamycin plates with respect to the wild type (Fig. 3f), confirming the involvement of *pvrR* in the regulation of phenotypic switching. However, because 100% of the variants expressing *pvrR* reverted to the wild-type phenotype, we propose that PvrR is implicated primarily in inducing reversion from variant to wild-type phenotypes. In addition, as PvrR overexpression reverts RSCV with 100% efficiency, it is unlikely that RSCV formation is mechanistically related to the high frequency hypermutability observed in *P. aeruginosa* CF isolates[14]. Finally, our results suggest that PvrR may be acting upstream of the phenotypic switch, as inactivation of *pvrR* by mutation does not result in conversion to the variant type.

The data presented in here indicate that *P. aeruginosa* is capable of undergoing transient phenotypic changes that allow the bacteria to increase their antibiotic resistance both *in vitro* and *in vivo*. Analogous to phase variation phenomena, antibiotic-resistant RSCV cells representing a relatively small fraction of the bacterial population ensure its survival in the presence of antibiotics. It remains to be determined whether the underlying mechanism of RSCV formation involves the types of DNA rearrangements that characterize other phase variable systems[11,15].

Because some of the phenotypes found in PA14 RSCV have been associated with the emergence of antibiotic resistance in bacterial biofilms[16,17], we speculate that resistant phenotypic variants present in *P. aeruginosa* biofilms are responsible for the increased resistance to antimicrobial agents observed in CF infections by *P. aeruginosa*. Moreover, we propose that variant phenotypes selected inside mature biofilms by antibiotic treatment and other conditions present in the lung of CF patients or in the biofilm itself (such as

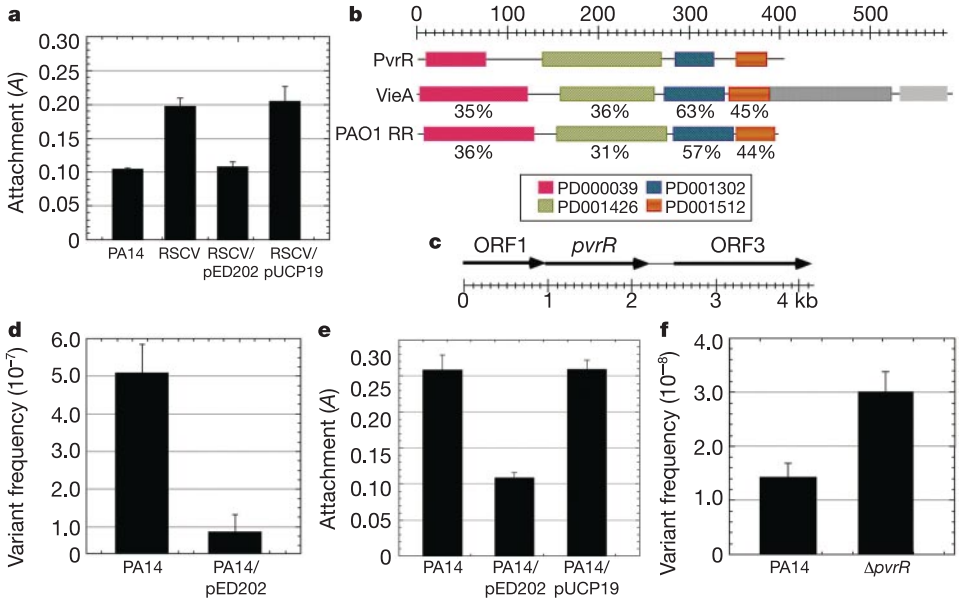

Figure 3 Identification and characterization of the *P. aeruginosa* PvrR response regulator. **a**, Attachment of strains to PVC plastic (see Methods). **b**, Alignment of PvrR-predicted amino-acid sequence with *V. cholerae* VieA and the *P. aeruginosa* PAO1 putative response regulator PA3947 (PAO1 RR). Numbers above scale indicate number of amino acids. The lower panel contains domain family numbers according to ProDom nomenclature. **c**, The *pvrR* gene is flanked by ORF1 and ORF3 with the same transcriptional orientation. **d**, Effect of PvrR overexpression on the frequency of variant formation. **e**, Effect of PvrR overexpression on attachment to PVC. **f**, Deletion of *pvrR* affects the frequency of variants.

nutrient limitation) constitute the so-called resistant biofilm phenotype. Identification of a two-component response regulator, PvrR, that modulates the phenotypic switch from antibiotic-resistant to antibiotic-susceptible forms and also regulates biofilm formation, reinforces the possibility that these mechanisms are interrelated. Importantly, our data suggest that activation of regulatory elements like PvrR could prevent biofilm formation and render bacteria more susceptible to antimicrobial agents. Because the appearance of phenotypic variants in response to antibiotic treatment has been reported in both Gram-negative and Gram-positive bacteria[18], resistance mechanisms similar to the one found in this study may be common among other bacterial pathogens. □

Methods

Bacterial strains, media and culture conditions

Clinical and CF *P. aeruginosa* isolates were provided by G. Pier or obtained from the Massachusetts General Hospital Clinical Microbiology Laboratory. M63 medium[19] was supplemented with 0.3% glucose, 1 mM $MgSO_4$ and 0.5% casamino acids (CAA). Modified FAB medium (0.1 mM $CaCl_2$, 0.01 mM Fe-EDTA, 0.15 mM $(NH_4)_2SO_4$, 0.33 mM Na_2HPO_4, 0.2 mM KH_2PO_4 and 1 mM $MgCl_2$) contained 0.5% CAA and 10 mM sodium citrate. Antibiotic concentrations (per ml) were: 100 μg ampicillin and 30 μg kanamycin for *Escherichia coli*; 300 μg carbenicillin for *P. aeruginosa*. We purchased antibiotics from Sigma.

Attachment and cell surface hydrophobicity assays

Attachment assays[20] were performed in 96-well PVC plates using M63 supplemented with CAA (0.5%), $MgSO_4$ (1 mM) and glucose (0.3%) or LB media. We determined hydrophobic surface properties using the salt aggregation test (SAT)[21].

Continuous-culture biofilm

Bacteria were cultivated in M63 in flow chambers with channel dimensions of 12 × 52 × 2 mm (provided by M. Franklin) for biofilm characterization, or in FAB medium in flow chambers with channel dimensions of 1 × 40 × 4 mm (Stovall) for measurement of biofilm resistance. The flow system was prepared and assembled as described[22]. Flow cells in both cases were inoculated with 100-fold dilutions of overnight cultures of PA14 or RSCV expressing GFP on plasmid pSMC21 (derivative of pSMC2 (ref. 23) provided by G. O'Toole). After inoculation, the medium flow was stopped for 1 h. Medium flow was resumed at a rate of 0.21 ml min^{-1} using a peristaltic pump (IsmaTec), and the flow-cell system was incubated at 37 °C. We used a Leica TCS SP system (Leica Lasertechnik; GmgH) for CSLM. Image analysis of antibiotic-treated biofilms was done in structures contained within serial section stacks of images delimited by freehand drawing. Pixel intensities unique to GFP-labelled bacteria and surrounding biofilm were established by the threshold limit technique. The volume (in μm^3) of individual biofilm structures was determined from serial sections using ImageSpace software (Molecular Dynamics).

Frequency of appearance of kanamycin-resistant variants

Frequency of appearance of kanamycin-resistant variants was determined by subculturing strains PA14 or PA14 carrying pED202 in LB liquid medium, and incubating for 15–16 h at 37 °C. Cultures were plated on LB, LB without NaCl or M63 containing kanamycin (200 μg ml^{-1}) after plates were dried for at least 1 h. Plates were incubated 36–40 h at 37 °C or 25 °C before resistant variants were counted.

Analysis of sputum samples from CF patients

Sputum samples were suspended in 5 ml of 10 mM $MgSO_4$. Serial dilutions of samples were plated onto Cetrimide agar (Difco) with and without antibiotics. Plates were screened for the presence of *P. aeruginosa* after 48 h of incubation at 37 °C. Colonies were later verified to be *P. aeruginosa* by probing colony lifts with the exotoxin A gene from *P. aeruginosa* (contained in the *Eco*RI–*Hin*dIII fragment of plasmid pRGI (ref. 24).

Antibiotic susceptibility of reverted variants

Variants obtained from CF samples were grown in 5 ml LB agar overnight at 37 °C. After incubation, serial dilutions of the bacterial suspension were plated onto antibiotic-free LB agar, and incubated overnight at 37 °C, and subsequently for 5 days at 25 °C. Plates were examined for the presence of revertants at the edges of the colonies. We repeated this process until stable revertants were obtained. *In vitro* susceptibility to the different antibiotics used was determined by establishing minimal bactericidal concentrations (MBCs) for the reverted variants as described[25].

Cloning of genetic region controlling phenotypic variation

A PA14 genomic library in vector pJSR1 (ref. 7) was mobilized en masse into RSCV PA14 using strain pRK2013 as helper[26]. Cosmid pED20 was subcloned into the pUCP19 plasmid vector[27] using a *Pst*I restriction digest.

Construction of a *P. aeruginosa pvrR* mutant

A *pvrR* deletion was generated by replacing 2.33 kb of the wild-type sequence containing the *pvrR* gene with a 1.416-kb fragment amplified by PCR. The PCR-amplified fragment was subcloned into the *Xba*I and *Sma*I sites of pCVD442 (ref. 28), generating pED167, which was introduced by allelic exchange into the homologous region of the PA14 chromosome as described[28].

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Competing interests statement

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Correspondence and requests for materials should be addressed to F.M.A. (e-mail: ausubel@molbio.mgh.harvard.edu). The sequences for *pvrR*, ORF1 and ORF3 are available at GenBank under accession number AF482691.