Phenotypic diversification *in vivo*: *Pseudomonas aeruginosa* *gacS*[−] strains generate small colony variants *in vivo* that are distinct from *in vitro* variants

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Pseudomonas aeruginosa has long been known to produce phenotypic variants during chronic mucosal surface infections. These variants are thought to be generated to ensure bacterial survival against the diverse challenges in the mucosal environment. Studies have begun to elucidate the mechanisms by which these variants emerge *in vitro*; however, too little information exists on phenotypic variation *in vivo* to draw any links between variants generated *in vitro* and *in vivo*. Consequently, in this study, the *P. aeruginosa* *gacS* gene, which has previously been linked to the generation of small colony variants (SCVs) *in vitro*, was studied in an *in vivo* mucosal surface infection model. More specifically, the rat prostate served as a model mucosal surface to test for the appearance of SCVs *in vivo* following infections with *P. aeruginosa* *gacS*[−] strains. As in *in vitro* studies, deletion of the *gacS* gene led to SCV production *in vivo*. The appearance of these *in vivo* SCVs was important for the sustainability of a chronic infection. In the subset of rats in which *P. aeruginosa* *gacS*[−] did not convert to SCVs, clearance of the bacteria took place and healing of the tissue ensued. When comparing the SCVs that arose at the mucosal surface (MS-SCVs) with *in vitro* SCVs (IV-SCVs) from the same *gacS*[−] parent, some differences between the phenotypic variants were observed. Whereas both MS-SCVs and IV-SCVs formed dense biofilms, MS-SCVs exhibited a less diverse resistance profile to antimicrobial agents than IV-SCVs. Additionally, MS-SCVs were better suited to initiate an infection in the rat model than IV-SCVs. Together, these observations suggest that phenotypic variation *in vivo* can be important for maintenance of infection, and that *in vivo* variants may differ from *in vitro* variants generated from the same genetic parent.

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INTRODUCTION

It has become increasingly evident that phenotypic diversification is a survival strategy that *Pseudomonas aeruginosa* employs. Under various laboratory conditions, *P. aeruginosa* produces phenotypic variants with altered colony morphologies (Boles *et al.*, 2004; Davies *et al.*, 2007; Déziel *et al.*, 2001; Kirisits *et al.*, 2005; Starkey *et al.*, 2009). Interestingly, the production of these variants appears to be related to the biofilm mode of growth; *P. aeruginosa* produces far larger proportions of colony variants in biofilms compared with planktonic cultures (Boles *et al.*, 2004). The promotion of variants in biofilms may, in part,

account for the observation that biofilms are inherently more resistant to antimicrobials than their planktonic (free-living) counterparts (Ceri *et al.*, 1999b; Costerton *et al.*, 1999). Indeed, given that variant production in biofilms is increased when exposed to environmental stressors, it is likely that variants play a role in community survival. *P. aeruginosa* variants have been shown to be more resistant than wild-type (WT) to heavy metals, oxidants and antibiotics (Boles *et al.*, 2004; Boles & Singh, 2008; Davies *et al.*, 2007; Harrison *et al.*, 2007).

Given the diversity and resistance of biofilms observed *in vitro*, it follows that phenotypic diversification may be a survival strategy *P. aeruginosa* adopts during mucosal surface infections, such as those in cystic fibrosis (CF) patients, which are characterized by biofilm formation (Singh *et al.*, 2000). Examinations of respiratory samples from CF patients with chronic *P. aeruginosa* infections tend to reveal numerous morphological variants, which supports this proposal (Drenkard & Ausubel, 2002; Govan & Deretic, 1996; Häussler *et al.*, 1999, 2003; Zierdt &

Abbreviations: C-di-GMP, cyclic di-GMP; CBD, Calgary Biofilm Device; CF, cystic fibrosis; CSLM, confocal scanning laser microscopy; Gro/CINC, growth-regulated oncogene/cytokine-induced neutrophil chemoattractant; IL, interleukin; IV-SCVs, *in vitro* small colony variants; MN, mononuclear; MPO, myeloperoxidase; MBEC, minimum biofilm eradication concentration; MS-SCVs, mucosal surface small colony variants; PMN, polymorphonuclear; SCVs, small colony variants; TGF, transforming growth factor; WT, wild-type.

Schmidt, 1964). The most common morphological variants isolated include small colony variants (SCVs) and mucoid colonies that overexpress alginate (Govan & Deretic, 1996; Häussler *et al.*, 1999). The presence of either of these variant types in sputa from CF patients correlates with decreased lung function and a poorer prognosis for the patient (Häussler *et al.*, 1999; Pedersen *et al.*, 1992; Schneider *et al.*, 2008). Moreover, SCVs are better able to form biofilms, adhere to lung epithelia, resist antibiotic treatment and, in some cases, are more cytotoxic to macrophages than WT isolates (Davies *et al.*, 2007; Drenkard & Ausubel, 2002; Häussler *et al.*, 2003; von Götz *et al.*, 2004). Together, these observations suggest that phenotypic variants play an important role in the disease process. Yet, it is unclear what triggers variant generation *in vivo*. Diversity may be initiated by stress from the host immune system, antibiotics prescribed to the patient or perhaps a combination of the two. Some recent work has begun to implicate antibiotics in variant production as long-term antibiotic use has correlated with the emergence of SCVs (Drenkard & Ausubel, 2002; Häussler *et al.*, 1999). It is likely that selection pressure from the host mucosal environment also contributes to variant production. However, studying phenotypic variation within a host is largely an unexplored avenue of research. For instance, the question remains whether the genetic pathways in *P. aeruginosa* that lead to phenotypic variation *in vitro* are the same pathways that produce variants *in vivo*.

In an exploration of this question, we chose to study phenotypic diversification within a host as mediated by the *P. aeruginosa* *gacS* (global activator of antibiotic and cyanide synthesis) gene. The *gacS* gene, along with *gacA*, constitutes an important two-component regulatory system involved in biofilm formation, environmental adaptation and chronic infections. Interestingly, it has been shown *in vitro* that stress applied to a *P. aeruginosa* *gacS*[−] strain results in phenotypic diversification of this strain into SCVs (Davies *et al.*, 2007). These SCVs are hyper-biofilm formers that are more resistant to antimicrobials than either the *gacS*[−] parent or WT bacteria. We hypothesized that stress from the host encountered at a mucosal infection site would likewise result in the production of SCVs from *P. aeruginosa* *gacS*[−] bacteria. These *in vivo*-derived SCVs were predicted to share the same characteristics as *in vitro* SCVs in terms of their increased biofilm formation and antimicrobial resistance.

In an examination of this hypothesis, we used our previously developed rat prostatitis model of acute and chronic *P. aeruginosa* infections as a model mucosal surface to test if *gacS*[−] bacteria would diversify to form SCVs under *in vivo* stress (Nelson *et al.*, 2009). A *P. aeruginosa* PA14 *gacS*[−] strain previously characterized by our laboratory was used to infect the rat prostate so that *in vivo*-derived SCVs produced by this mutant would be comparable with *in vitro* SCVs from the same parent (Davies *et al.*, 2007). By using the rat prostate model in conjunction with the PA14 *gacS*[−] mutant we show that diversity generation can be critical for maintaining an infection *in vivo*. Furthermore, we show that SCVs that arise *in vivo* exhibit both similarities to and differences from *in vitro* SCVs derived from the same *gacS*[−] parent.

METHODS

Bacteria, media and culture conditions. *P. aeruginosa* PA14 strains used and isolated in this study are summarized in Table 1. Notably, PA14 mucosal surface-derived SCVs (MS-SCVs) were recovered from both acute and chronic PA14 *gacS*[−] infections. For antimicrobial susceptibility testing, MS-SCVs recovered from acute and chronic infections behaved identically, and therefore data are shown only for the acute MS-SCV isolate. All strains were stored at −70 °C in Microbank vials (Pro-Lab Diagnostics) according to the manufacturer's instructions. To create an inoculum for the animal model, second *P. aeruginosa* subcultures grown on Miller Luria-Bertani (LB; Difco) agar were used to establish a culture of 1×10^6 cells (ml saline)^{−1}, as determined by McFarland standards. Bacterial numbers were later reconfirmed by plate counts on both LB and Pseudomonas isolation agar (PIA; Difco). *P. aeruginosa* isolated from the animal model following infection was cultured on both LB and PIA. For antimicrobial susceptibility testing, all PA14 strains were grown and cultured on trypticase soy broth and trypticase soy agar (TSB and TSA, respectively; Difco).

Animal model. The rat prostate served as a model for mucosal surface infections and was infected as described previously (Ceri *et al.*, 1999a; Nelson *et al.*, 2009; Nickel *et al.*, 1990; Phan *et al.*, 2008; Rippere-Lampe *et al.*, 2001). Briefly, 300 g male Sprague–Dawley rats were obtained from the Life and Environmental Sciences Animal Resource Centre at the University of Calgary. The rats were maintained in polycarbonate box cages on aspen chip bedding and were provided rat chow and water *ad libitum*. Cages were housed at 20 ± 2 °C and 40 ± 10 % relative humidity with 12 h of daily illumination. During the infection procedure, rats were anaesthetized with halothane and catheterized through the urethra with a lubricated sterile PE10 polyethylene feeding tube. *P. aeruginosa* inocula, prepared as above, were injected into the catheter at a volume of

Table 1. Bacterial strains used in this study

Strain	Genotype or description	Source
PA14 WT	Wild-type <i>P. aeruginosa</i>	Rahme <i>et al.</i> (1995)
PA14 <i>gacS</i> [−]	PA14Δ <i>gacS</i> ::gm ^r	Davies <i>et al.</i> (2007)
PA14 IV-SCV	PA14Δ <i>gacS</i> ::gm ^r , <i>in vitro</i> -derived small colony variant	Davies <i>et al.</i> (2007)
PA14 MS-SCV	PA14Δ <i>gacS</i> ::gm ^r , mucosal-surface derived small colony variant recovered from PA14 <i>gacS</i> [−] prostate infection	This study

0.2 ml with the catheter placed at the base of the prostate. Four *P. aeruginosa* (PA14) strains were used to infect the rat prostate: WT, *gacS*, *in vitro*-derived SCVs (IV-SCVs) and MS-SCVs. The PA14 *gacS*[−] strain was instilled into 16 rats for both the acute and the chronic models (*n*=32). All other PA14 strains (WT, IV-SCV and MS-SCV) were instilled into eight rats for both the acute and the chronic models (*n*=48). The prostate was infected with *P. aeruginosa* for the lengths of time that most accurately emulate the pathology seen in human acute and chronic prostate infections (2 and 12 days, respectively), after which rats were killed by CO₂ asphyxiation (Ceri *et al.*, 1999a, c; Nelson *et al.*, 2009). Approval for this study was granted by The Life and Environmental Sciences Animal Care Committee and all animal experiments were performed in accordance with guidelines of the Canadian Council of Animal Care.

Tissue preparation. The ventral prostate was aseptically removed from the rat, photographed and weighed. The prostate tissue was then cut into four sections. One piece was homogenized in sterile saline to determine bacterial counts per milligram of tissue. The remaining three pieces were processed for histology, myeloperoxidase (MPO) assays and cytokine analysis, as described in the following sections.

Histological sections. In accordance with previous studies, prostate tissue fixed in 10 % (v/v) neutral-buffered formalin was embedded in paraffin, cut to sections of 5 µm thickness and stained with standard haematoxylin and eosin (H&E) protocols (Nelson *et al.*, 2009; Phan *et al.*, 2008). Sections were scored blindly according to the criteria established by a veterinary anatomical pathologist, as described previously (Nelson *et al.*, 2009). Acute sections (2 days) were scored as follows: Grade 0, a normal appearance; Grade 1, oedema and infiltrates of polymorphonuclear (PMN) leukocytes in the prostate stroma; Grade 2, PMN leukocytes in the prostate acini; Grade 3, loss of epithelial and/or basement membrane architecture in the prostate acini; Grade 4, haemorrhage; and Grade 5, tissue necrosis. Chronic sections (12 days) were graded as microscopically showing: Grade 0, a normal appearance; Grade 1, oedema and PMN or mononuclear (MN) leukocytes in prostate stroma; Grade 2, PMN or MN leukocytes in the prostate acini; Grade 3, loss of epithelial and/or basement membrane architecture in the prostate acini; Grade 4, necrosis accompanied by PMN and MN leukocytes and/or fibrosis; and Grade 5, necrosis with no evidence of tissue repair and absence of PMN and MN leukocytes.

Myeloperoxidase assay. Prostate tissue was homogenized in hexadecyltrimethyl ammonium bromide (HTAB) buffer followed by centrifugation at 13 000 *g* for 2.5 min. Supernatants were mixed with *O*-dianisidine in phosphate buffer in a 96-well microtitre plate, followed by immediate readings of optical density at 450 nm (OD₄₅₀) by using a TERMOMax microplate reader with Softmax Pro data analysis software (Molecular Devices). MPO activity was recorded per milligram of protein present in the prostate sample (Mullane *et al.*, 1985).

Cytokine [interleukin (IL)-1β, growth-regulated oncogene/cytokine-induced neutrophil chemoattractant (Gro/CINC)-1, IL-10 and transforming growth factor (TGF)-β] assays. Tissues to be screened for cytokine levels were homogenized in PBS containing 2 µl protease inhibitor cocktail ml^{−1} (Sigma). The homogenates were centrifuged at 1000 *g* for 10 min and supernatants were used for cytokine assays. Assays were performed by using commercial ELISA kits according to the manufacturer's instructions for rat IL-10, TGF-β, Gro/CINC-1 (R&D systems) and IL-1β (eBiosciences).

Biofilm formation. *In vitro* biofilms of *P. aeruginosa* were grown in the Calgary Biofilm Device (CBD) (Innovotech) according to the methods previously developed in our laboratory (Ceri *et al.*, 1999b). In brief, *P. aeruginosa* strains of interest were cultured twice on TSA from cryogenic stocks. Colonies from the second subculture were used to create a starting inoculum of 10⁷ c.f.u. ml^{−1} by suspending

colonies in 0.9 % (w/v) saline to match a 1.0 McFarland standard, followed by a 30-fold dilution in TSB. A 150 µl aliquot of this inoculum was then placed into each well of a 96-well microtitre plate, into which the sterile lid of the CBD was placed. The lid of the CBD contains 96 pegs on which biofilms grow following placement in the inoculum. Biofilm formation on the peg was facilitated by incubation at 37 °C with 95 % relative humidity on a gyratory shaker at 125 r.p.m. Following incubation of the inoculated device for 24 h, pegs from the device were collected for either confocal scanning laser microscopy (CSLM), for antimicrobial susceptibility testing or to evaluate biofilm cell density. To evaluate biofilm cell density, pegs were rinsed in 0.9 % (w/v) saline, broken off the lid with sterile pliers and placed in 0.9 % (w/v) saline containing Tween 20 (1 %, v/v). Pegs were sonicated for 30 min in an Aquasonic model 250HT ultrasonic cleaner (VWR Scientific) to disrupt the biofilms from the pegs (Harrison *et al.*, 2010). To ensure biofilms were removed from the pegs, four sonicated pegs from each strain grown in the CBD were visualized by CSLM. In all cases, biofilms were eradicated from the pegs and only a few adherent bacterial cells remained (data not shown). The biofilms removed from the pegs were serially diluted in 0.9 % (w/v) saline and plated on TSA for viable cell counts.

CSLM. The 3D structure of biofilms was evaluated by CSLM. Pegs that were broken from the CBD were stained with 0.05 % (w/v) acridine orange (Sigma) for 5 min. The fluorescently labelled biofilms were examined by using a Leica DM IRE2 spectral confocal and multiphoton microscope with a Leica TCS SP2 acoustic optical beam splitter (Leica Microsystems), as described previously (Davies *et al.*, 2007; Harrison *et al.*, 2006). All images of biofilm slices were captured by using a ×63 water-immersion objective. Three-dimensional reconstruction of the confocal image stacks was accomplished by using IMARIS v6.3.1 software (Bitplane).

Stock solution of antimicrobials and neutralizers. Antibiotics, metals, 30 % (w/v) hydrogen peroxide (H₂O₂) and hypochlorite (OCI[−]) were purchased from Sigma. Stock solutions of antibiotics were made to a concentration of 5120 µg ml^{−1} by using double-distilled water (ddH₂O) as a dilutant, followed by storage in 0.5 ml aliquots at −70 °C. Metals were also diluted to starting concentrations in ddH₂O, but were syringe-filtered and stored at room temperature prior to use. H₂O₂ and OCI[−] were diluted directly from the bottle supplied by the manufacturer immediately before use in the challenge plate. Challenge plates were made containing the antimicrobial of interest in Mueller Hinton broth (Difco) 30 min prior to use. For challenge plates containing metals, universal neutralizer was added to the recovery media (TSB, 1 % Tween 20, v/v). For the universal neutralizer, reduced glutathione (GSH) and L-cysteine were prepared at 0.25 M in ddH₂O, syringe-filtered and stored at −20 °C.

Antimicrobial susceptibility testing. Antibiotic, metal and biocide susceptibility testing was performed on the *P. aeruginosa* biofilms that had grown on the lid of the CBD as previously described (Ceri *et al.*, 1999b; Davies *et al.*, 2007; Harrison *et al.*, 2004, 2010). All *P. aeruginosa* strains were challenged with antimicrobials after 24 h biofilm growth. At 24 h, the lid of the device was rinsed in 200 µl of 0.9 % (w/v) saline and then placed in a microtitre plate containing serial, twofold dilutions of the antimicrobial of interest (the 'challenge plate'). The first and last rows of the challenge plate did not contain any antimicrobial and were used as either sterility controls or growth controls, respectively. After 24 h exposure to the challenge plate, the lid of the device was removed, rinsed twice in 0.9 % (w/v) saline and placed in 200 µl of recovery media (TSB, 1 % Tween 20, v/v). Biofilms were disrupted in the recovery medium by sonication in an ultrasonic cleaner. Following this, aliquots of 20 µl from the wells of the recovery medium were serially diluted tenfold in 0.9 % (w/v) saline and plated on TSA. Viable cells surviving antimicrobial exposure were enumerated on TSA plates that were incubated for 24 h at 37 °C.

Statistical analysis. Group data are expressed as mean $\pm$ SEM. Figures and statistical analyses were compiled by using GraphPad Prism 5 software. Bacterial counts and MPO readings were $\log_{10}$ -transformed. Following this, bacterial counts, MPO assays and cytokine ELISAs were analysed by one-way ANOVA and Tukey's multiple comparison test. P -values <0.05 were considered statistically significant.

RESULTS

Appearance of *gacS*⁻ SCVs *in vivo*

Since *P. aeruginosa gacS*⁻ generates SCVs under stressful *in vitro* conditions (Davies *et al.*, 2007), we believed the same would hold true under *in vivo* stress. Consequently, the rat prostate was infected with PA14 *gacS*⁻ and the appearance

of SCVs in prostate tissue was monitored for acute and chronic infections (2 and 12 days post-infection, respectively) (Fig. 1). Notably, the total number of *gacS*⁻-infected rats differed between acute and chronic rats ($n=12$ vs $n=14$) as catheter leakage occurred from two rats in the acute group, disqualifying them from analysis.

SCVs were isolated from prostate homogenates for the majority of *gacS*⁻-infected rats; 67 and 71 % of rats were positive for SCVs for acute and chronic infections, respectively. In acute infections, SCVs comprised a mean of 13 % of the bacteria isolated, whereas in chronic infections 24 % of the isolated colonies were SCVs. By means of comparison, in rats infected with PA14 WT ($n=8$) no SCVs were isolated. Whereas SCVs arose *in vivo* for the majority of rats infected with *P. aeruginosa gacS*⁻, it

Fig. 1. Bacterial colonization and prostate inflammation in rat prostates infected with *P. aeruginosa* PA14 strains. Solid grey bars represent *gacS*⁻-infected rats. This group was divided into two subgroups: one in which SCVs were produced from *gacS*⁻ bacteria (checked) and one where no SCVs were isolated (striped). Black bars denote SCV-infected rats. Bacterial numbers in prostate tissue (a, e), weight of prostate (b, f), MPO levels in prostate tissue (c, g) and IL-1β levels in prostate tissue (d, h) during acute (a–d) and chronic (e–h) infections. Horizontal bars indicate a statistical difference between groups ($P < 0.05$). For (a–d) and (e–h), respectively, *gacS*⁻, $n=12$ or 14; *gacS*⁻ with SCV, $n=8$ or 10; *gacS*⁻ without SCV, $n=4$.

was interesting to note that SCVs failed to be produced in approximately 30 % of rats in both acute and chronic infections. When subsequent measures of bacterial counts and inflammation were performed, bacterial survivability and infectious capability were significantly reduced when SCVs were not present (Fig. 1). In the subset of rats in which SCVs were not present, bacteria were nearly eradicated from the prostate in both acute and chronic infections (Fig. 1a, e). In contrast, when SCVs were isolated from the prostate, bacterial counts similar to WT levels were found (Fig. 1a, e). Similar trends were observed when inflammation was assessed by prostate weight (as a measure of oedema) and MPO levels (as a measure of inflammatory cell recruitment) (Mullane *et al.*, 1985). Comparing rats which differed in SCV isolation, significantly lower prostate weight and MPO levels were seen in the non-SCV-producing group in both acute and chronic infections (Fig. 1b, c, f, g).

Tissue damage in rats infected with PA14 *gacS*[−] and WT bacteria was scored to assess whether the appearance of SCVs correlated with the severity of tissue damage. As emergence of SCVs correlates with decreased lung function and weight loss in CF patients (Häussler *et al.*, 1999; Schneider *et al.*, 2008), we reasoned that mucosal tissue damage would be more pronounced in cases where SCVs were isolated from prostate homogenates. In line with this, the majority of rats scored in the moderate to severe range of tissue damage when SCVs were isolated from either acute or chronic infections (Fig. 2). In stark contrast, no rats scored in this range when SCVs were not isolated from acute infections (Fig. 2a). In chronic infections, in the absence of SCVs, some rats did score in the moderate range, but this represented only 20 % of the rats infected (Fig. 2b).

Effect of *gacS*[−] SCV infection on mucosal surfaces

Since prostate infection was more apparent in cases where SCVs were produced from *P. aeruginosa gacS*[−], we hypothesized that a mucosal surface infection consisting strictly of SCVs would result in considerable infection and inflammation. Therefore, a set of rats were infected with

gacS[−] SCVs that were isolated from two different sources: the prostate mucosal surface (MS-SCV) and a biofilm grown *in vitro* (IV-SCV). We used both the MS-SCV- and the IV-SCV-derived SCVs to infect the rat prostate to see if pathogenicity was altered based on the source of SCV. Interestingly, differences were seen between IV-SCV and MS-SCV in acute infections only (Figs 1 and 2). In acute infections, IV-SCV prostate infections produced significantly lower bacterial counts, prostate weight and MPO levels than MS-SCV infections (Fig. 1a–c). Surprisingly, for all measures of infection and inflammation, IV-SCV infections were statistically equivalent to *gacS*[−] infections that did not produce any SCVs (Fig. 1a–c). Similar trends were seen for tissue damage where histological signs of inflammation were mostly absent in IV-SCV rats (Fig. 2a). In chronic infections, no differences were observed between IV-SCVs and MS-SCVs, both of which caused statistically higher inflammation and tissue damage than the *gacS*[−] non-SCV-producing group (Figs 1e–g and 2b). Of note, MS-SCVs and WT did not differ statistically with regard to inflammatory levels, except for measures of prostate weight and MPO levels in acute infections where MS-SCVs surpassed WT (Fig. 1b, c).

As an additional measure of inflammation in SCV-infected rats, both pro- and anti-inflammatory cytokine levels in rat prostate tissue were determined. The pro-inflammatory cytokines that were chosen for assessment were selected based on the fact that they are well-established markers of inflammation in the rat prostate (Nelson *et al.*, 2009; Phan *et al.*, 2008). Consistent with our previous observations, the pro-inflammatory cytokine IL-1 β was elevated in acute WT and MS-SCV infections in comparison with IV-SCV- and non-SCV-producing groups (Fig. 1d). As typical for IL-1 β , levels were reduced considerably in chronic infections, such that differences between groups were no longer noticeable (Fig. 1h). A second pro-inflammatory cytokine, Gro/CINC-1, showed similar trends to IL-1 β ; however, here differences between groups did not reach statistical significance (data not shown). Lastly, levels of the anti-inflammatory cytokines IL-10 and TGF- β were measured; levels were relatively consistent among groups, indicating that these cytokines do not play a role in the response to *P. aeruginosa gacS*[−] or *P. aeruginosa* SCVs (data not shown).

Fig. 2. Percentage of rats that displayed moderate to severe inflammation (graded as 3, 4 or 5 out of a score of 5) following prostate infection with *P. aeruginosa* PA14 strains. Prostate sections were processed, stained and graded as described in Methods. (a) Acute infections, (b) chronic infections.

Biofilm formation of *gacS*⁻ SCVs from mucosal surface infections

Biofilm formation has been shown to vary between WT, *gacS*⁻ and *in vitro*-derived SCV *P. aeruginosa* morphotypes. IV-SCVs isolated from *P. aeruginosa* form exceptionally dense biofilms, whereas parental *gacS*⁻ biofilms form very sparse and thin biofilms (Boles *et al.*, 2004; Davies *et al.*, 2007; Drenkard & Ausubel, 2002; Häussler *et al.*, 2003). Thus, we hypothesized that dense biofilms would likewise be produced by the MS-SCVs isolated from the prostate. To test this, PA14 IV-SCVs, as well as PA14 WT, *gacS*⁻ and MS-SCVs isolated from prostate homogenates, were grown in the CBD for 24 h and subsequently visualized by CSLM. At 24 h, similar cell densities of 5.5 ± 0.4 , 5.7 ± 0.5 , 6.0 ± 0.7 and 6.1 ± 0.6 log₁₀ c.f.u. per peg were seen for PA14 WT, *gacS*⁻, IV-SCV and MS-SCV biofilms, respectively. However, the distribution of cell density was markedly different; *P. aeruginosa gacS*⁻ formed flat microcolonies across the peg whereas WT and SCV strains displayed a concentrated biomass at the air–liquid interface. As a result, biofilm density varied between strains. Representative biofilms visualized with CSLM from each strain are shown in Fig. 3. WT *P. aeruginosa* formed biofilms that displayed some degree of complexity (Fig. 3a), whereas biofilms from *gacS*⁻ strains sparsely covered the surface (Fig. 3b). IV-SCVs formed dense biofilms with complicated 3D structure (Fig. 3c); these were similar to MS-SCV biofilms (Fig. 3d). Biofilms of MS-SCVs reached a

height of 40 µm, about two and four times the height of WT and *gacS*⁻ biofilms, respectively.

Antimicrobial susceptibility of *gacS*⁻ SCVs from mucosal surface infections

A number of studies have found that SCVs, whether isolated from *in vitro* biofilms or from CF sputum, are more resistant to antimicrobials than the WT (Davies *et al.*, 2007; Drenkard & Ausubel, 2002; Häussler *et al.*, 1999). Conversely, *P. aeruginosa gacS*⁻ is particularly susceptible to antimicrobials (Davies *et al.*, 2007). Thus, it was hypothesized that the MS-SCVs isolated from the prostate would be more resistant to antimicrobial agents than PA14 WT or *gacS*⁻. Additionally, antimicrobial susceptibility of IV-SCVs was evaluated to determine if differences existed between *in vivo* and *in vitro* SCVs derived from the same *gacS* parent.

Antimicrobial susceptibility was evaluated by performing susceptibility testing on biofilms of PA14 WT, *gacS*⁻, IV-SCV and MS-SCV grown in the CBD for 24 h as similar cell densities were seen at this time point (see biofilm results above). The anti-biofilm activity of four antimicrobial agents, H₂O₂, OCl⁻, tobramycin and Ag⁺, was tested against each of these four strains (Table 2 and Fig. 4). Bactericidal concentrations of the antimicrobial agents were defined as the minimum biofilm eradication concentration required to kill 99.9% of the bacteria (MBEC_{99.9}). This definition is consistent with the American Clinical and

Fig. 3. Biofilm formation of *P. aeruginosa* PA14 wild-type (a), *gacS*⁻ (b), IV-SCVs (c) and MS-SCVs (d). Biofilms were grown in TSB in the CBD, stained with acridine orange and imaged by using CSLM. The 3D reconstructions (created by using IMARIS v6.3.1 software) and side-views of each biofilm are shown.

Table 2. Biofilm antimicrobial susceptibility of *P. aeruginosa* PA14 WT, *gacS*[−], IV-SCV and MS-SCV strains against various antimicrobials

MBEC_{99.9}, minimum biofilm eradication concentration required to kill 99.9 % of the bacteria. Values are the mean ± SD of data from four independent trials.

Antimicrobial	MBEC _{99.9}			
	WT	<i>gacS</i> [−]	IV-SCV	MS-SCV
H ₂ O ₂ (%)	1.80 ± 2.17	0.26 ± 0.27	6.25 ± 2.50	5.00 ± 0.00
OCl [−] (mM)	0.16 ± 0.06	0.34 ± 0.19	2.50 ± 1.00	2.25 ± 1.26
Ag ⁺ (mM)	0.60 ± 0.42	0.34 ± 0.19	4.8 ± 0.00	0.41 ± 0.53
Tobramycin (μg ml ^{−1})	28.00 ± 26.53	14.00 ± 4.00	36.0 ± 20.13	28.00 ± 8.00

Laboratory Standards Institute's definition of a bactericidal concentration of a 3 log₁₀ reduction in viable bacterial cells (<http://www.clsi.org/>). The MBEC_{99.9} values of each of the antimicrobial agents against the WT, *gacS*[−], IV-SCV and MS-SCV strains are summarized in Table 2. As expected, and consistent with previously published work, the MBEC_{99.9} values were considerably higher for IV-SCVs than for WT and *gacS*[−] for all antimicrobials tested. Results for MS-SCVs were similar to those for IV-SCVs for the oxidants H₂O₂ and OCl[−]. Unlike IV-SCVs, however, biofilms of MS-SCVs did not show any increased resistance to the heavy metal Ag⁺. The same held true for Cu²⁺; IV-SCVs were highly resistant to this heavy metal, but MS-SCVs and *gacS*[−] isolates were very susceptible to killing (data not shown). Finally, whereas MS-SCVs were more resistant to tobramycin than the *gacS*[−] isolate, MBEC_{99.9} values were the same for MS-SCVs and WT.

When resistance of biofilms from PA14 *gacS*[−], IV-SCVs and MS-SCVs was examined against various antimicrobial concentrations, reproducible patterns of resistance were observed (Fig. 4). In general, with the exception of Ag⁺, MS-SCVs were far more resistant to antimicrobials than *gacS*[−] strains (Fig. 4). MS-SCVs and IV-SCVs were equally resistant to antimicrobials at all antimicrobial concentrations tested, except that MS-SCVs succumbed at a much lower concentration of Ag⁺ (Fig. 4). As illustrated by heavy metals, the source of SCV (mucosal surface or *in vitro*) affected the resistance profile. Therefore, *gacS*[−] colonies isolated from the prostate were also tested for their antimicrobial resistance to see if they exhibited an altered resistance pattern from the original *gacS*[−] strain used to infect the prostate. However, in all cases, *gacS*[−] strains isolated from the prostate showed the same resistance pattern as the infecting PA14 *gacS*[−] strain (data not

Fig. 4. Susceptibility of *P. aeruginosa* *gacS*[−] (●), IV-SCV (▲) and MS-SCV (■) strains to antimicrobials. Biofilms were exposed to various antimicrobials as detailed in Methods. Killing of biofilms against (a) H₂O₂, (b) OCl[−], (c) tobramycin and (d) Ag⁺.

shown). Thus, MS-SCV biofilms were resistant to H₂O₂ and OCl⁻ oxidative stressors, but not to heavy metals; *gacS*⁻ biofilms were highly susceptible to all antimicrobials tested; and IV-SCV biofilms were, under the conditions tested here, universally resistant.

DISCUSSION

In humans, mucosal surfaces are often the target of opportunistic *P. aeruginosa* infections. It has been proposed that successful colonization of these mucosal environments, which present numerous and heterogeneous challenges, necessitates the evolution and diversification of bacteria (Oliver *et al.*, 2000; Rainey & Travisano, 1998). The fact that numerous phenotypic variants of *P. aeruginosa* have been recovered from patients with mucosal infections supports this proposal (Govan & Deretic, 1996; Häussler *et al.*, 1999, 2003; Zierdt & Schmidt, 1964). However, although these variants have been well characterized, factors leading to their production at the mucosal surface are not well understood. As such, it becomes difficult to speculate if mechanisms attributed to phenotypic variation *in vitro* are also at play *in vivo*. In this study, we addressed this issue by examining a gene (*gacS*) previously linked to *P. aeruginosa* variant production *in vitro* (Davies *et al.*, 2007) to determine its ability to also produce variants *in vivo*. The *gacS* gene was chosen for two reasons. First, SCVs produced from deletions of the *gacA* and *gacS* genes have been well characterized *in vitro* (Davies *et al.*, 2007; Parkins *et al.*, 2001). This allows for a thorough comparison with the *in vivo* SCVs generated from *gacS*⁻ bacteria in this study. Secondly, unlike WT bacteria which rarely produce stable SCVs, exposure of *gacS*⁻ bacteria to *in vitro* stress causes stable SCVs to be generated with a high frequency. These SCVs exhibit characteristics similar to other SCVs described in the literature, including resistance to antimicrobials and dense biofilm formation (Boles *et al.*, 2004; Boles & Singh, 2008; Kirisits *et al.*, 2005; Starkey *et al.*, 2009). Therefore, we believed that *gacS*⁻ SCVs produced *in vivo* would be representative of *P. aeruginosa* SCVs.

Having selected *gacS*⁻ bacteria for use in our *in vivo* infection model (Nelson *et al.*, 2009), we found that in the majority of rats infected with *gacS*⁻ bacteria, MS-SCVs were produced. However, in approximately 30% of animals, no MS-SCVs were isolated. The amount of colonizing bacteria, the level of inflammation and the extent of tissue damage in animal tissues were markedly different between the two groups, showing that infection is severely compromised when SCVs were not produced from a *gacS*⁻ strain (Figs 1 and 2). Considering that neither acute nor chronic infections were very successful in the absence of MS-SCV, it is curious that PA14 *gacS*⁻ bacteria failed to convert to SCVs in as many as 30% of animals. Given that the input signals that GacS responds to are unknown, as are the mechanisms by which *P. aeruginosa* *gacS*⁻ enhances SCV production, it is difficult to speculate

on the reason for SCV appearance in some, but not all, PA14 *gacS*⁻-infected animals. As future research addresses these signalling pathways, it may become clearer why SCVs are produced in the infection model. Additionally, clues may be offered through recent and ongoing research concerning other genes and molecules linked to SCV production. For instance, the intracellular signalling molecule cyclic di-GMP (c-di-GMP) has recently been implicated in SCV production (Meissner *et al.*, 2007; Starkey *et al.*, 2009). Mutations in the *wspF* gene constitutively activate the diguanylate cyclase WspR, which elevates c-di-GMP levels and subsequently converts a WT *P. aeruginosa* strain into SCVs. Additionally, depletion of c-di-GMP in a *wspF* mutant strain converts the SCV back to WT morphology (D'Argenio *et al.*, 2001; Hickman *et al.*, 2005). The extrapolysaccharide biosynthetic locus has also been implicated through the *pel* and *psl* gene clusters, which are required for the SCV phenotype (Starkey *et al.*, 2009). Thus, SCV production in an infection, or lack thereof, may be a result of various signalling pathways. Regardless, in any of these possible pathways, it is hypothesized that there is a race to convert *gacS*⁻ bacteria to the more successful SCV form. In cases where the possible signal is recognized and the switch to the SCV phenotype occurs, the infection progresses to a typical chronic prostatitis; when the recognition of the switch does not occur, the *gacS*⁻ cells are not able to withstand the host immune response, and therefore bacterial clearance and healing of the prostate takes place.

Although the reasons for SCV production in select *gacS*⁻ prostate infections cannot yet be accounted for, it can be said that when MS-SCVs were produced, colonization and inflammation occurred at the mucosal surface (Figs 1 and 2). Likewise, when MS-SCVs infected the prostate, markers of colonization and inflammation were noticeably present in both acute and chronic infections (Figs 1 and 2). Interestingly, apart from measures of prostate weight and MPO in acute infections, MS-SCV infections were similar to WT in terms of virulence (Fig. 1b, c). This response is slightly different from that reported by Starkey *et al.* (2009), which showed that clinical and laboratory SCVs from other genetic backgrounds were less immunostimulatory than WT. The difference could reflect that the study by Starkey *et al.* (2009) was performed on human epithelial cell lines for 4 h, whereas the current study was undertaken in a rat mucosal surface model for a period of either 2 or 12 days. It could also be a phenomenon particular to MS-SCVs in a *gacS*⁻ background where these SCVs match the WT in their immune stimulation. In either case, the data suggest that MS-SCV production in a *gacS*⁻ mutant produces an infection comparable with WT and therefore the *gacS* gene is not necessarily required for an infection. This would stand in contrast with the other member of the GacS–GacA two-component system, the *gacA* gene, which is required for virulence in both acute and chronic infections in numerous model systems (Chieda *et al.*, 2005; Coleman *et al.*, 2003; Mahajan-Miklos *et al.*, 1999; Rahme *et al.*, 2000; Tan *et al.*, 1999). This difference is

probably accounted for by the fact that, unlike *gacS*⁻ mutants, *gacA*⁻ mutants do not produce stable SCVs (Parkins *et al.*, 2001).

It is clear that SCVs facilitate infection (Figs 1 and 2) and that the GacS system is involved in their production. When the *gacS* gene is supplied back to a SCV isolate from a *gacS*⁻ strain, the SCV reverts back to its larger colony phenotype (Davies *et al.*, 2007). Additionally, *P. aeruginosa* *gacS*⁻ strains produce stable SCVs at a high frequency *in vitro* (Davies *et al.*, 2007) and *in vivo*, as shown in this work (13 and 24% of bacteria in the prostate are MS-SCVs in acute and chronic infections, respectively). As such, the question becomes: could losing the *gacS* gene in an effort to facilitate high-frequency production of SCVs be advantageous? The present data suggest that this may be the case as the *gacS* gene seems to represent a fitness trade-off. While the *gacS*⁻ mutants were highly susceptible to antimicrobial agents, this cost was counter-balanced by the production of MS-SCVs that were exceptionally more resistant to antimicrobial stressors than WT (Table 2 and Fig. 4). Additionally, MS-SCVs were generally similar to WT in terms of virulence (Fig. 1) and therefore MS-SCVs were not easily cleared nor excessively damaging to the host, neither of which would benefit survivability of the bacteria. Together, this implies that a deletion in *gacS* affords *P. aeruginosa* the ability to produce a high frequency of phenotypic variants that can still maintain infection and strongly resist antimicrobials. Moreover, the MS-SCVs produced from *gacS*⁻ strains were very stable, which would mean a resistant population would remain even when the initial stressor was removed. Further supporting that a deletion of the *gacS* gene could offer a survival advantage, studies with *Pseudomonas fluorescens* and *Pseudomonas chlororaphis* have shown that mutations in *gacS* are often selected for during phenotypic diversification of these pseudomonads (Sánchez-Contreras *et al.*, 2002; Schmidt-Eisenlohr *et al.*, 2003). However, a cautionary note in interpreting *gacS*⁻ mutations as favourable in the current study is required, as it is acknowledged that the MS-SCVs arising from *gacS*⁻ mutants may have accumulated other genetic mutations that contributed to the observed phenotype. Also, it is worth noting that *gacS*⁻ mutations at the onset of infection do not appear overly advantageous, as *gacS*⁻ bacteria were cleared in 30% of rats in the study (Fig. 1). A survival advantage may be more likely if the *gacS* mutations accumulated over time as they would in a natural infection. In this case, the clearance rate of bacteria may be considerably lower than the one observed in this study where all infecting bacteria already possessed the *gacS* deletion.

Subsequent to determining that MS-SCVs arise *in vivo*, these MS-SCVs were compared with *in vitro* SCVs from the same *gacS*⁻ parent. This comparison was undertaken as there has previously been some question as to whether laboratory-derived and clinically derived variants are comparable. Although altered phenotypes between CF and laboratory SCVs were detected by von Götz *et al.* (2004), others have

shown that motility traits and transcriptional profiles between the two types of SCVs were similar (Kirisits *et al.*, 2005; Starkey *et al.*, 2009). To our knowledge, this study is the first to investigate this issue by comparing an *in vitro* (IV-SCV) and *in vivo* (MS-SCV) -derived variant arising from the same genetic mutation. When comparing the IV-SCVs and MS-SCVs derived from PA14 *gacS*⁻ bacteria, it was revealed that they behaved similarly, in that both were able to cause infection (Figs 1 and 2), form dense biofilms (Fig. 3) and resist killing by antimicrobials (Table 2 and Fig. 4). Despite these general similarities, there were notable differences. One clear difference between IV-SCVs and MS-SCVs was their behaviour in acute infections. IV-SCVs and MS-SCVs were equally adept at causing chronic infections, but IV-SCVs were impaired in their ability to cause acute infections (Figs 1 and 2). These data may suggest that passage through a mucosal infection creates MS-SCVs that are capable of colonization and inflammation immediately upon reinfection. In contrast, IV-SCVs can only establish a chronic infection given sufficient time to adapt as they lacked the benefit of a previous exposure to the host system.

In addition to their ability to cause infection, MS-SCVs and IV-SCVs also differed in the specificities of their antimicrobial resistance. Whereas IV-SCVs showed increased resistance to numerous heavy metals, antibiotics and oxidants, MS-SCVs appeared to be selectively resistant. MS-SCVs were resistant to H₂O₂ and OCl⁻, but were similar to WT in terms of their eradication threshold to tobramycin and were not at all resistant to heavy metals (Table 2 and Fig. 4). Thus, MS-SCVs were not indiscriminately resistant to antimicrobials, but rather were resistant to the oxidants H₂O₂ and OCl⁻, which they would have encountered through exposure to host neutrophils. Given that MS-SCVs were not resistant to silver and copper, which also kill by inducing oxidative stress (Harrison *et al.*, 2009), it appears that MS-SCVs are resistant to host-specific oxidative stressors. As such, oxidative stressors in the host may prompt phenotypic variation. It has previously been shown that oxidative stress selects for diversification *in vitro*, and it has since been speculated that oxidative stress similarly causes phenotypic variation *in vivo* (Boles & Singh, 2008). Our results support this hypothesis. They further suggest that previous exposure to the immune system is not necessarily sufficient for increased resistance to oxidants present in neutrophils as the *gacS*⁻ colonies recovered from the prostate were not resistant to H₂O₂ and OCl⁻ (Table 2 and Fig. 4). Therefore, it is likely that the properties of MS-SCVs, such as dense biofilm formation (Fig. 3), are responsible for the increased resistance to neutrophil stressors. Apart from the oxidative stressors present in neutrophils, antibiotics may also be playing a role in the selection process. Unlike the MS-SCVs studied here, SCVs isolated from CF patients are considerably more resistant to tobramycin than WT counterparts (Drenkard & Ausubel, 2002; Häussler *et al.*, 1999). Tobramycin is an antibiotic commonly prescribed

to CF patients, and therefore SCVs isolated from these patients may have been selected for because of their increased tolerance to tobramycin. Overall, it would appear that SCVs arising from infection sites exhibit resistance to the stressors they encountered. MS-SCVs differ from IV-SCVs in this regard as IV-SCVs appear to be resistant to most antimicrobials, regardless of the initial stressor that selected the IV-SCV (Foglia *et al.*, 2005). It is currently unclear why IV-SCVs appear to exhibit resistance to most antimicrobials whereas MS-SCVs do not. It is possible that laboratory conditions encourage resistance to numerous stressors whereas host conditions are more likely to select for resistance to a particular stressor. Nevertheless, we can conclude that given the dissimilarities in resistance profiles between MS-SCVs and IV-SCVs, especially in terms of metal tolerance, *in vivo* and *in vitro* variants from the same parent can be physiologically different.

Overall, these differences between MS-SCVs, IV-SCVs and *gacS*⁻ strains begin to reveal the importance of *in vivo* diversification for *P. aeruginosa* and the ways in which it differs from observations made *in vitro*. However, future studies revealing the genetic mechanisms underlying these differences are required to fully understand the processes involved in successful adaptation to the host.

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