

# Role of small colony variants in persistence of *Pseudomonas aeruginosa* infections in cystic fibrosis lungs

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**Abstract:** *Pseudomonas aeruginosa* is an opportunistic pathogen that predominates during the later stages of cystic fibrosis (CF) lung infections. Over many years of chronic lung colonization, *P. aeruginosa* undergoes extensive adaptation to the lung environment, evolving both toward a persistent, low virulence state and simultaneously diversifying to produce a number of phenotypically distinct morphs. These lung-adapted *P. aeruginosa* strains include the small colony variants (SCVs), small, autoaggregative isolates that show enhanced biofilm formation, strong attachment to surfaces, and increased production of exopolysaccharides. Their appearance in the sputum of CF patients correlates with increased resistance to antibiotics, poor lung function, and prolonged persistence of infection, increasing their relevance as a subject for clinical investigation. The evolution of SCVs in the CF lung is associated with overproduction of the ubiquitous bacterial signaling molecule cyclic-di-GMP, with increased cyclic-di-GMP levels shown to be responsible for the SCV phenotype in a number of different CF lung isolates. Here, we review the current state of research in clinical *P. aeruginosa* SCVs. We will discuss the phenotypic characteristics underpinning the SCV morphotype, the clinical implications of lung colonization with SCVs, and the molecular basis and clinical evolution of the SCV phenotype in the CF lung environment.

**Keywords:** small colony variants, cystic fibrosis, cyclic-di-GMP, *Pseudomonas aeruginosa*, RsmA, antibiotics

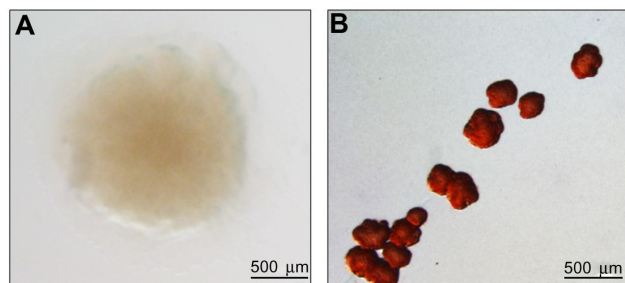
## Introduction

Cystic fibrosis (CF) is a recessively inherited genetic disease in which the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene is mutated, leading either to partial or complete loss-of-function. *CFTR* encodes a chloride ion channel, and the loss of ion transport across epithelial cell membranes leads to osmotic imbalance, and consequently to the buildup of sticky mucus in the lower respiratory tract.<sup>1</sup> CF lungs are highly prone to microbial infection, with chronic infection beginning in infancy for the overwhelming majority of cases and continuing throughout the patient's life. *Pseudomonas aeruginosa* is an opportunistic Gram-negative pathogen and the predominant infective species isolated from late-stage CF lung infections.<sup>2,3</sup> Once established in the lungs of CF patients, *P. aeruginosa* infections are extremely difficult to completely eradicate, and the aggravation and progressive tissue degradation associated with repeated infectious relapses lead to morbidity and eventually death.<sup>3</sup> *P. aeruginosa* lung infections typically progress clonally from infection with a single environmentally acquired genotype<sup>4,5</sup> and, over the course of long-term chronic CF infections, undergo extensive genetic and phenotypic adaptation to the lung environment.<sup>6,7</sup>

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There are two main consequences of this adaptation: first, the progressive transition toward a persistent, low virulence state, and second, a related diversification into a number of different phenotypes.<sup>6,7</sup> These include mucoid cells, which overproduce alginate and form distinctive slimy colonies,<sup>8</sup> and small colony variants (SCVs, Figure 1), which are typically slow-growing isolates that show strong attachment to surfaces, autoaggregation, enhanced exopolysaccharide production, and biofilm formation.<sup>9,10</sup> In recent years, several studies have established a causal link between *P. aeruginosa* SCVs and persistence of infection in animal models,<sup>11–13</sup> supporting the hypothesis that the SCV phenotype confers a fitness advantage under chronic infection conditions, and thus plays an important role in the pathogenesis of *P. aeruginosa* lung infections. *P. aeruginosa* SCVs also emerge in other situations that favor chronic infections, including in mechanically ventilated patients or in those suffering from chronic obstructive pulmonary disease.<sup>14,15</sup> These studies suggest that persistent *P. aeruginosa* morphotypes such as SCV represent genetic adaptations to the hostile milieu in the patient, with characteristics including resistance to phagocytosis,<sup>12</sup> antimicrobial resistance due to slow growth or increased persister cell populations,<sup>16,17</sup> and reduced virulence<sup>18</sup> potentially contributing to selection.

In this review, we will summarize the current state of research into clinical *P. aeruginosa* SCVs. We will discuss the initial discovery and characterization of SCVs in the sputum of CF patients, the phenotypic characteristics associated with the SCV morphotype, and the clinical implications of lung colonization with SCVs. Finally, we will address the molecular basis of the SCV phenotype and possible evolutionary routes to SCV generation, with an emphasis on SCV evolution in the lung environment.



**Figure 1** The *Pseudomonas aeruginosa* SCV phenotype.

**Notes:** Photographs of *P. aeruginosa* strains grown on LB agar containing 0.004% Congo Red dye for 24 hours at 37°C. **(A)** Wild-type *P. aeruginosa* PAO1 forms flat, slightly wrinkled colonies. **(B)** The SCV strain  $\Delta yfiR^{13}$  forms small, rugose colonies that adhere strongly to surfaces and bind tightly to Congo Red. Photographs courtesy of Sebastian Pfeilmeier.

**Abbreviations:** LB, Lysogenic broth; SCV, small colony variant.

## Discovery and initial characterization of SCVs in the CF lung

Phenotypic variation between different *P. aeruginosa* isolates in CF sputum has been recognized for many years. While the alginate-overproducing mucoid phenotype<sup>8</sup> has until recently garnered the most attention, it has long been recognized that SCVs (previously known as dwarf colonies) also arise in the chronically infected respiratory tract of CF patients.<sup>19</sup> For example, Thomassen et al<sup>20</sup> examined *P. aeruginosa* isolates from 286 CF patients over a 3-month period in 1976–1977 and determined the frequency and distribution of different morphotypes. In this study, dwarf colonies were identified in the sputum of 14% of patients, although these colonies were always found in conjunction with other morphotypes.<sup>20</sup> The first clinical study to specifically focus on the distribution and phenotypic characteristics of *P. aeruginosa* SCV isolates from CF patients took place in Germany from 1996 to 1998. Häussler et al<sup>17</sup> tested the sputum of 86 CF patients for *P. aeruginosa* SCVs, identifying them in 33 patients. Over the course of this study, around 3.0% of the total *P. aeruginosa* isolates collected were classed as SCVs. These colonies were characterized as small (1–3 mm in diameter), slow growing, and more resistant to several different classes of antibiotics than subsequent planktonic revertants.<sup>17</sup> Häussler et al<sup>17</sup> went on to characterize some of their SCV isolates, reporting that these strains were hyperpilated and autoaggregative. The SCV morphs tested showed increased twitching motility and a marked ability to form biofilms and to attach to human pneumocytes.<sup>10</sup> Simultaneously with these clinical SCV studies, the molecular biology of SCV formation was being investigated by two independent groups. Drenkard and Ausubel<sup>21</sup> showed that *P. aeruginosa* SCV formation could be induced under laboratory conditions by the addition of kanamycin and linked this phenotype to the putative cyclic-di-GMP (cdG) phosphodiesterase gene *pvrR*. At the same time, D'Argenio et al<sup>22</sup> identified the WspR diguanylate cyclase (DGC) and showed that it was responsible for SCV formation in the laboratory strain PAO1.

## Phenotypic characteristics of the SCV morphotype

In agreement with the early clinical research, clinical and laboratory-derived SCVs generally display increased resistance to a broad range of antibiotics.<sup>17,21,23</sup> Exposure to sub-inhibitory antibiotic concentrations has been shown to induce attachment and SCV formation in vitro, possibly as part of

a defense response.<sup>21,24</sup> SCVs are also usually nonmotile, with flagellar motility absent in most SCVs characterized to date.<sup>12,23,25</sup> Hyperpiliation has been reported for some strains, with these isolates displaying increased twitching motility as a result,<sup>10</sup> although the distribution of this phenotype is unknown. Probably the most prominent phenotype associated with SCV formation is the significantly enhanced production of one or more exopolysaccharide molecules.<sup>9,12,25</sup> *P. aeruginosa* produces at least three exopolysaccharides. The most noticeable of these in clinical CF isolates is alginate, the overproduction of which produces the slimy, mucoid phenotype.<sup>8</sup> The role of alginate in SCV formation is unclear, although SCV strains have been identified that overproduce this molecule.<sup>26</sup> Also produced are the glucose-rich exopolysaccharide Pel, and Psl, which contains glucose, mannose, and rhamnose.<sup>27</sup> Both Pel and Psl overproduction have been explicitly linked to SCV formation, and deletion of both *pel* and *psl* operons reverts an SCV to a smooth phenotype.<sup>12,25</sup> Many of the other phenotypes associated with SCV, including their enhanced biofilm formation and surface attachment,<sup>10</sup> small size (stemming from autoaggregation),<sup>17</sup> resistance to phagocytosis,<sup>12</sup> and infectious persistence,<sup>11–13</sup> have been linked to exopolysaccharide overproduction in these morphs.

A striking characteristic of *P. aeruginosa* in the CF lung is its extraordinary degree of phenotypic diversity. For example, based on a year-long study of sputum samples from ten CF patients infected with the same initial *P. aeruginosa* strain, Mowat et al<sup>28</sup> examined the phenotypic characteristics of 1,720 individual isolates. The *P. aeruginosa* populations in each individual sputum sample in this study showed extensive phenotypic diversity, with the majority of diversity occurring within sputum samples rather than between patients. In agreement with this, Workentine et al<sup>29</sup> examined a more extensive series of phenotypes for 169 clonal isolates from a single chronically colonized patient. Once again, the researchers observed a very high degree of phenotypic variation. Interestingly, every isolate in this study presented a different phenotypic profile from the others, with very little correlation seen between the majority of tested phenotypes.<sup>29</sup>

The implications of these findings for SCVs in the lung environment are that apart from the core phenotypic characteristics by which SCVs are identified in the clinic, a high degree of variation might be expected for every other phenotype. Examination of the phenotypes of clinical SCVs has largely confirmed this hypothesis. For example, Häussler et al<sup>17</sup> reported reduced growth rates for their clinical SCV

isolates, an observation that has since been repeated for other SCV genotypes growing in liquid media.<sup>23</sup> However, work from other researchers has shown that this does not apply to all strains or under all growth conditions.<sup>30</sup> Similarly, siderophore production has been reported as being downregulated in some SCVs,<sup>30</sup> but as being upregulated in others.<sup>12,23,31</sup> Cytotoxicity/virulence is another potentially plastic phenotype in SCVs. Long-term lung colonization is generally associated with a loss of cytotoxicity, and downregulation of the type III secretion system (T3SS).<sup>32</sup> Reduced cytotoxicity is a recognized characteristic of *rsmA*- strains,<sup>13</sup> which form SCVs under laboratory conditions.<sup>33</sup> However, a transcriptional analysis of the well-studied strain SCV20265 showed significant upregulation of T3SS genes compared to its clonal predecessor.<sup>31</sup> This increased expression agreed with the results of an earlier proteomic analysis of the same strain<sup>34</sup> and corresponded to significantly increased cytotoxicity in a murine infection model.<sup>31</sup> Given that SCVs generally arise after significant periods of lung colonization, many of the phenotypes associated with long-term, chronic lung infection (loss of virulence, reduced quorum sensing, hypermutability, etc<sup>18,23,35–37</sup>) might be expected to correlate with SCV. However, as the studies above indicate, many exceptions exist, and phenotypic variation between SCV morphotypes is likely to be extensive.<sup>28,29</sup>

## The clinical implications of SCV lung infection

At least two independent reports have identified a strong relationship between the presence of SCVs in the CF lung and poor clinical outcomes. The 1999 Häussler et al<sup>17</sup> study of SCVs in CF sputum examined a total of 86 patients with chronic *P. aeruginosa* lung infections. While only 3% of all colonies recovered during the study were classed as SCVs, those patients from whom SCVs were isolated (33 out of 86) displayed poorer lung function than those who did not display SCV colonization. The forced expiratory volume in the first second (FEV<sub>1</sub>) score for these patients was 56%, which was significantly lower than the FEV<sub>1</sub> of SCV-negative patients (80%). More recently, Schneider et al<sup>38</sup> looked for *P. aeruginosa* and *Staphylococcus aureus* SCVs in sputum samples from 98 CF patients. Over a 3-month period, 9.2% of patients in this study were colonized with *P. aeruginosa* SCVs.<sup>38</sup> In agreement with the findings of Häussler et al,<sup>17</sup> patients having SCVs had poorer lung function, with lower blood oxygen levels and a significantly lower FEV<sub>1</sub> score (39%) than the non-SCV patients (65.5%). The body mass of SCV-colonized patients was also significantly lower

(mean 14.0% underweight compared with 2.7% for non-SCV patients).<sup>38</sup>

While it is difficult to confidently infer a causal relationship between the SCV phenotype and poor clinical prognosis based on correlation studies alone, there is substantial evidence supporting a role for SCVs in persistence in a clinical setting. Three independent research groups, using different animal infection models and genetically distinct SCV strains, have established causal relationships between the SCV phenotype and persistence of infection.<sup>11–13</sup> Furthermore, many of the phenotypes associated with SCV have been directly linked to advantageous survival traits during chronic infection. For example, Malone et al<sup>12</sup> showed that extracellular polysaccharides (EPS) overproduction rendered *P. aeruginosa* SCVs highly resistant to macrophage phagocytosis. As mentioned previously, SCVs generally show enhanced antibiotic resistance compared to wild-type *P. aeruginosa*.<sup>17,21,23</sup> While a causal relationship again remains to be established, this antibiotic resistance may contribute to SCV survival in the CF lung during antibiotic chemotherapy.

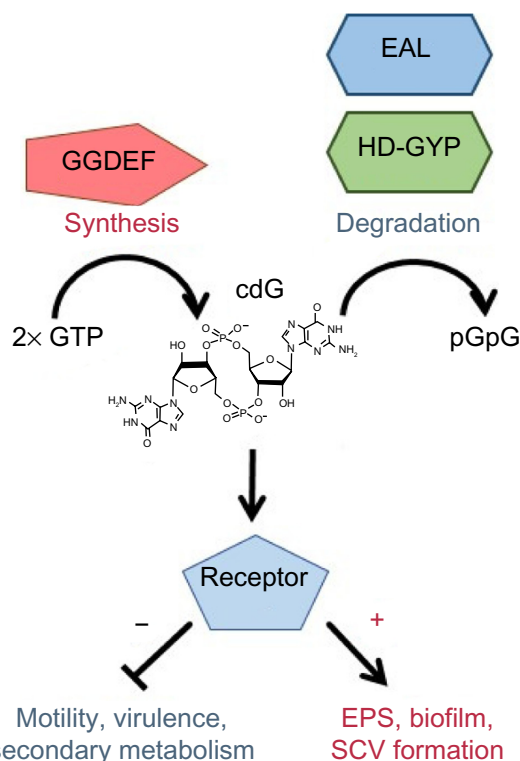
Fully defining the relationship between SCVs and persistence in the CF lung is likely to remain a challenge for the foreseeable future. While correlations between clinical SCV colonization and poor lung function are clear, and SCVs contribute to infectious persistence in laboratory and animal studies, it is currently unknown whether SCVs trigger poor clinical outcomes or just preferentially associate with deteriorating lungs. Short of invasive or postmortem investigations of CF lungs, an interesting recent advance that may provide an answer is the ex vivo porcine lung model developed by Harrison et al.<sup>39</sup> This system has been successfully tested with *P. aeruginosa* quorum sensing mutants and promises to shed further light onto microbial colonization of the lung environment.

## The molecular bases of the SCV phenotype: cyclic-di-GMP and the GacAS/RsmAZY pathway

Following early indications from two independent research labs,<sup>21,22</sup> strong evidence has accumulated for a causal link between the SCV phenotype and the bacterial second messenger cdG.<sup>12,25,40–42</sup> cdG is a ubiquitous bacterial signaling molecule that controls a wide range of cellular processes involved in the transition between motile, virulent, and sessile biofilm forming lifestyles.<sup>43,44</sup> The cyclic dinucleotide is produced from two molecules of GTP (guanosine triphosphate) by DGCs and degraded to pGpG (5'-phosphoguanylyl-(3'-5')-guanosine) by

phosphodiesterases;<sup>45</sup> enzymes containing the conserved GGDEF and EAL/HD-GYP domains, respectively.<sup>46–49</sup> In general, cdG production is associated with community behavior phenotypes such as EPS production and biofilm formation, while low cdG levels lead to enhanced motility, virulence, and a single-celled lifestyle<sup>43</sup> (Figure 2). cdG signal transduction is a highly complex process, with many bacterial species containing dozens of different cdG-signaling proteins.<sup>43</sup> *P. aeruginosa* is no exception, with 33 predicted GGDEF and 17 EAL domain-containing proteins.<sup>50,51</sup> These cdG metabolic enzymes control the intracellular level of cdG and hence regulate the expression of various phenotypic outputs. In *P. aeruginosa*, this includes exopolysaccharide production,<sup>52–54</sup> production and deployment of proteinaceous adhesins,<sup>55,56</sup> siderophore production,<sup>12</sup> rhamnolipid biosynthesis,<sup>57</sup> and virulence and cytotoxicity systems,<sup>51,58–60</sup> as well as the assembly, function, and control of type IV pili<sup>61,62</sup> and the bacterial flagellum.<sup>54,63–65</sup>

cdG affects cell behavior by controlling phenotypic outputs at every regulatory level, from gene expression through to allosteric modulation of phenotypic outputs.<sup>43</sup> These effects



**Figure 2** cdG signaling.

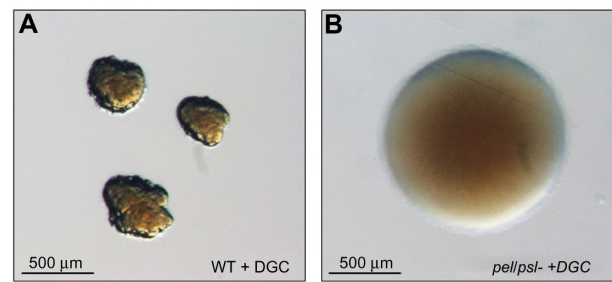
**Notes:** cdG is produced from GTP by GGDEF proteins and degraded by EAL and HD-GYP phosphodiesterases. cdG binds to a variety of different receptors, suppressing motility and virulence and promoting EPS production, biofilm formation, and the SCV phenotype.

**Abbreviations:** cdG, cyclic-di-GMP; EPS, extracellular polysaccharides; GTP, guanosine triphosphate; pGpG, 5'-phosphoguanylyl-(3'-5')-guanosine; SCV, small colony variant.

occur through binding to specific effector proteins, whose activity is altered upon interaction with cdG. Consistent with the high complexity of cdG signal transduction, binding is highly promiscuous and occurs with a wide range of different protein folds.<sup>66,67</sup> For example, the PilZ domain is a dedicated cdG binding protein, with examples like Alg44 in *P. aeruginosa*, which is a regulator of alginate biosynthesis.<sup>52</sup> Furthermore, not all GGDEF, HD-GYP, and EAL domain proteins have cdG metabolic activity. A substantial minority have degenerate active sites and instead function by binding cdG, via protein–protein interactions or through other alternative mechanisms.<sup>66,67</sup> For example, the *P. aeruginosa* EPS synthase component PelD binds to cdG, at a conserved site on its degenerate GGDEF domain, and induces a conformational change in the Pel synthase machinery that leads to activation and EPS production.<sup>68</sup> cdG also binds to a number of different transcriptional regulators. In *P. aeruginosa*, this includes the flagellar and EPS-gene master regulator FleQ, which controls *pel* and *psl* EPS operon transcription.<sup>54,69</sup> The metabolism of cdG is under extensive spatial and temporal control in *P. aeruginosa*. Expression and translation of cdG genes is regulated by various cellular inputs, including sigma factors, cell cycle control, quorum sensing, and translational regulation.<sup>43,60,70</sup> A further, significant level of control exists at the posttranslational level, with the majority of GGDEF, EAL, and HD-GYP domain proteins also containing diverse sensory inputs, including PAS, GAF, and response-regulator receiver domains.<sup>43</sup>

The role of cdG in *P. aeruginosa* SCV formation is now well-established. For example, a strong SCV phenotype can be triggered under laboratory conditions by overexpressing a DGC gene in *trans*,<sup>44</sup> a phenotype that can be reversed by disrupting the *pel* and *psl* EPS synthase operons (Figure 3).<sup>12</sup> The intracellular level of cdG has been shown to be elevated in several clinical and laboratory-derived SCVs.<sup>12,25,42</sup> Furthermore, mutations in several *P. aeruginosa* cdG signaling pathways have been shown to induce SCV phenotypes in vitro. In each case, elevated intracellular levels of cdG have been linked to overproduction of either exopolysaccharides<sup>9,25,41</sup> or fimbrial adhesins,<sup>40,71</sup> and consequently to SCV formation.

As well as cdG signaling pathways, other genetic loci have been implicated in persistence of infection and/or SCV formation in *P. aeruginosa*. A prominent example is the GacAS/RsmAZY signaling system (Figure 4), an important regulator of the switch between chronic and acute infectious lifestyles in many Gram-negative bacterial species, including *P. aeruginosa*.<sup>70,72</sup> RsmA is a small, translational regulator that specifically recognizes and binds to GGA sequences in the 5'

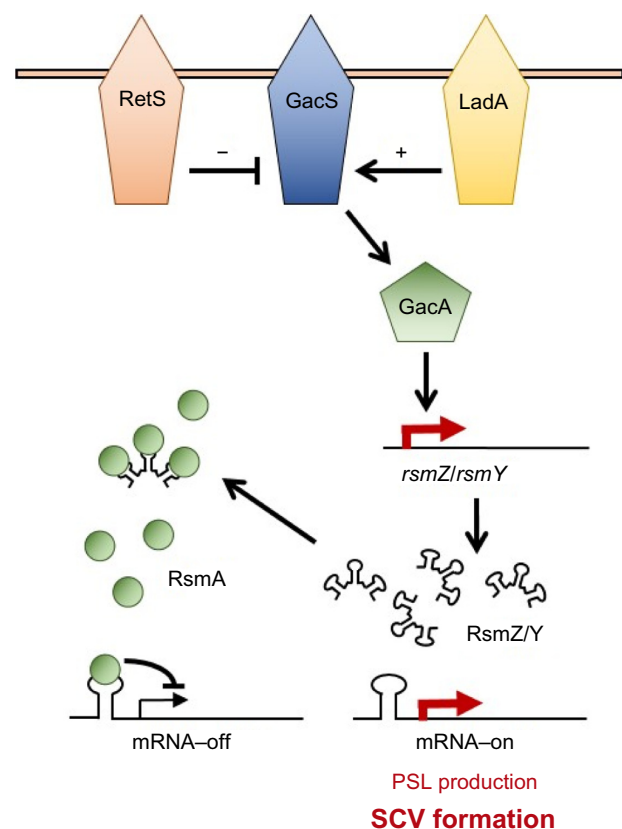


**Figure 3** cdG and EPS define the SCV phenotype.

**Notes:** Photographs of *Pseudomonas aeruginosa* strains grown on LB agar for 24 hours at 37°C. **(A)** Overexpression of the DGC gene *wspR* induces the SCV phenotype in *P. aeruginosa* PAO1. This phenotype is dependent on the presence of intact EPS operons. **(B)** Overexpression of *wspR* in a *pel/psl*- mutant still leads to alterations in colony morphology, but the distinctive small, rugose SCV phenotype is abolished. Photographs courtesy of Sebastian Pfeilmeier.

**Abbreviations:** cdG, cyclic-di-GMP; EPS, extracellular polysaccharides; SCV, small colony variant; LB, Lysogenic broth; DGC, diguanylate cyclase; WT, wild-type.

leader region of target mRNAs. RsmA reciprocally controls the translation of mRNAs associated with Type III/Type VI secretion and Psl exopolysaccharide synthesis,<sup>33,70</sup> leading to the suppression of biofilm-associated phenotypes and promoting secondary metabolism, motility, and virulence.



**Figure 4** The GacAS/RsmAZY signaling network.

**Notes:** GacAS-mediated expression of the sRNAs, RsmZ and RsmY, inactivates RsmA, leading to increased translation of target mRNAs, including for the Psl EPS pathway. GacS activity is controlled in turn by the hybrid TCS proteins LadA and RetS.

**Abbreviations:** EPS, extracellular polysaccharides; TCS, two-component signal.

Deletion of the *rsmA* gene in *P. aeruginosa* PA01 induces an SCV phenotype as a consequence of increased *psl* mRNA translation,<sup>33</sup> and has been shown to increase infectious persistence in a murine lung model.<sup>13</sup>

In *Pseudomonas* spp. the activity of RsmA is controlled by the GacAS two-component signal transduction system. GacS is a transmembrane histidine protein kinase and activates its cognate response regulator GacA by phosphotransfer. Phosphorylated GacA promotes transcription of the antagonistic sRNAs, RsmY and RsmZ.<sup>73</sup> RsmY/RsmZ contain multiple GGA trinucleotides in the exposed stem-loops of their predicted secondary structures and inhibit RsmA activity by competing for RsmA binding, thus titrating the translational regulator away from its target mRNAs (Figure 4). The GacAS system is itself controlled by the two-component signal hybrid proteins LadS and RetS. RetS is an antagonist of GacS activity, and thus suppresses RsmZ/Y levels. Rather than operating via a conventional histidine protein kinase phosphotransfer mechanism, RetS forms heterodimers with GacS, and thus blocks GacS auto-phosphorylation phosphotransfer to GacA.<sup>74</sup> The LadS protein positively controls *rsmZ/Y* expression, and thus works to suppress RsmA activity. Although the mechanism of LadS function is currently uncharacterized, it may work by counteracting RetS activity (Figure 4).<sup>72</sup> Signal transduction through the Gac/Rsm pathway is highly complex, and affects the production of dozens of different proteins.<sup>70</sup> In addition to direct RsmA translational regulation, the Gac/Rsm system indirectly affects numerous additional cellular behaviors via the regulation of signaling pathways, including both cdG metabolism and quorum sensing.<sup>60,70</sup> Similar to cdG, mutations in quorum sensing genes are strongly associated with persistence during chronic CF lung infections.<sup>6,75</sup>

## Evolution of SCVs in the CF lung

Having colonized the respiratory passage and lungs of a CF patient, in most cases, a *P. aeruginosa* infection persists throughout the patient's lifetime. Patients are typically colonized by a single environmentally acquired genotype<sup>4,5</sup> (although some genotypes such as the Liverpool epidemic strain are infectious and can be transmitted from patient to patient<sup>76</sup>). *P. aeruginosa* CF lung infections generally alternate between periods of chronic, largely asymptomatic colonization and relapses into aggravated infection.<sup>1</sup> Over several years, the progressive tissue degradation that accompanies these repeated infectious relapses leads to lung failure and premature death.<sup>3</sup> As stated above, *P. aeruginosa* strains undergo extensive adaptation to the lung environment

throughout the period of chronic lung infection,<sup>6,7</sup> with multiple distinct phenotypes including SCVs arising in the lung population.<sup>7,10,77</sup> Despite generally originating from a single clonal genotype, significant genetic variation exists between individual sputum isolates sampled at any one time,<sup>28,29</sup> providing an explanation for the long-observed phenotypic heterogeneity in CF *P. aeruginosa* isolates.<sup>19,20</sup>

Perhaps unsurprisingly, the most commonly identified SCV-inducing mutations are loss-of-function mutations in repressor proteins that control the activity of DGCs (Table 1). A well-studied example is the Wsp pathway,<sup>41</sup> which contains a methyl-accepting chemotaxis receptor (WspA) and a DGC response regulator (WspR). WspR overproduction/activation induces an SCV phenotype,<sup>22,41</sup> displaying strong attachment and increased expression of the *pel* and *psl* exopolysaccharide operons. Under laboratory conditions, the principle route to SCV evolution is via loss-of-function mutations in the methyl-esterase gene *wspF*.<sup>25,41</sup> Without WspF, WspA and hence WspR are constitutively activated, leading to cdG synthesis and SCV

**Table 1** Mutational targets for *Pseudomonas aeruginosa* SCV formation

Gene	Protein function	Notes	Reference
<i>yfiN</i>	DGC	Constitutively active mutants isolated from the CF lung	Malone et al <sup>26</sup>
<i>yfiR</i>	YfiN repressor protein	Putative loss-of-function mutants isolated from CF lung	Malone et al <sup>26</sup>
<i>wspF</i>	Methylesterase	Loss-of-function leads to activation of WspR DGC. Mutants identified in CF sputum isolates	Smith et al, <sup>6</sup> Blanka et al <sup>42</sup>
<i>fleQ</i>	Transcriptional regulator of <i>pel</i> and <i>psl</i> EPS loci	Mutation leads to SCV phenotype in vitro Mutants identified in CF sputum isolates	Smith et al <sup>6</sup>
<i>accBC</i>	Fatty acid biosynthesis operon	Upstream mutation stabilizes <i>accBC</i> mRNA, modifying plasma membrane and triggering WspR DGC activation	Blanka et al <sup>42</sup>
<i>mutS</i>	Mismatch repair system	Mutations arise with high frequency in CF lungs. Associated with persistence, chronic lifestyles, and SCV formation	Mena et al, <sup>35</sup> Oliver et al, <sup>37</sup> Hogardt et al <sup>79</sup>
<i>rsmA</i>	Translational regulator of chronic lifestyle switch	Mutation leads to SCV phenotype in vitro, possibly <i>mutS</i> associated?	Irie et al <sup>33</sup>

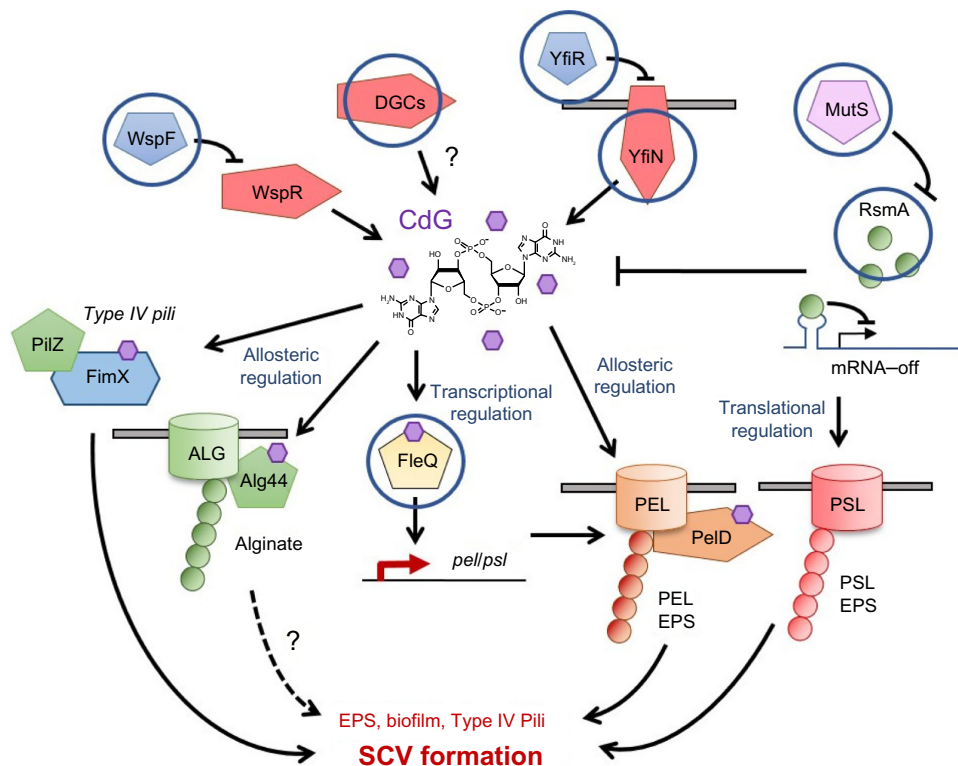
**Abbreviations:** SCV, small colony variant; DGC, diguanylate cyclase; CF, cystic fibrosis; EPS, extracellular polysaccharides.

formation through EPS overproduction (Figure 5).<sup>41,78</sup> There is evidence that *wspF* loss-of-function also represents a route to clinical SCV evolution. In 2006, Smith et al<sup>6</sup> carried out a longitudinal study of lung-adapted *P. aeruginosa* isolates and identified several different backgrounds in which the *wspF* gene was mutated. This study also identified a number of mutations in the transcriptional regulator *fleQ*. Deletion of *fleQ* induces an autoaggregative phenotype in PA01, with many of the characteristics of SCV colonies.<sup>54</sup> While the morphologies of the lung isolates in the Smith et al<sup>6</sup> study were not characterized in detail, these data nonetheless implicate both *wspF* and *fleQ* as potential mutagenic targets for SCV generation in the CF lung (Figure 5).

Blanka et al<sup>42</sup> recently identified an interesting additional mechanism for SCV evolution in the CF lung isolate SCV20265. Comparison of the SCV20265 genome with those of several laboratory-derived revertants identified a causal mutation for the SCV20265 aggregative phenotype. This study showed that a point mutation in the 5' untranslated region of *accBC*, a gene cluster responsible for fatty acid biosynthesis, leads to mRNA stabilization and a consequent increase in the proportion of short-chain fatty acids

in the plasma membrane. In turn, this change in membrane composition triggered activation of the Wsp system and cdG overproduction via WspR.<sup>42</sup> Intriguingly, the genome of SCV20265 was also shown to contain a second, additive mutation in the methyltransferase gene *wspF*, shown in previous studies to induce an SCV phenotype.<sup>6,25</sup> These findings suggest that both regulatory and genetic inputs combine to control cdG production, and hence SCV evolution, in the CF lung.<sup>42</sup>

The *yfiBNR* signaling operon<sup>12</sup> represents a further, well-characterized genetic target for clinically-arising SCVs. YfiN is a membrane-bound DGC whose activity is normally allosterically repressed by the soluble periplasmic repressor YfiR. Release of YfiR repression, either through a loss-of-function mutation in *yfiR* or an “escape” mutation in *yfiN*, leads to DGC activation, cdG overproduction, and SCV formation under laboratory conditions.<sup>12,26</sup> As with *wspF* and *fleQ*, activating mutations arise in the *yfi* locus (in both *yfiR* and *yfiN*) during long-term CF lung infections, driving SCV formation in vivo (Figure 5).<sup>26</sup> Interestingly, further examination of the CF isolates included in this study identified mutational “scars” in the *yfi* genes of two independent clinical CF lines.



**Figure 5** Mutational routes to SCV formation.

**Notes:** The diagram shows part of the cdG/RsmA signaling network in *Pseudomonas aeruginosa*. Hypothetical SCV-inducing mutations in uncharacterized DGCs are included. Signaling systems where mutations have been implicated in clinically relevant SCV formation are circled. Activating signals are denoted with arrows, suppression with bars. Purple hexagons represent cdG molecules. Pathways where the link to SCV is hypothetical or uncertain are marked with question marks (?). The dashed arrow refers to a pathway where the relationship to clinical SCV formation is currently unclear.

**Abbreviations:** SCV, small colony variant; DGC, diguanylate cyclase.

These strains contained both activating and loss-of-function mutations in the *yfiN* DGC gene, supporting the idea that YfiN activity is under both positive and negative selection in vivo, with Yfi-SCVs acting as an environmental pool for the generation of new smooth morphotypes in the complex and heterogeneous fitness landscape of the CF lung.<sup>26</sup>

Another class of mutations associated with the clinical emergence of the SCV phenotype are those in *mutS* and other mismatch repair genes. These hypermutable strains arise with high frequency in the course of chronic CF lung infection.<sup>37,79</sup> Mena et al<sup>35</sup> showed that a *P. aeruginosa* mismatch-repair mutation increased the long-term persistence of oropharyngeal colonization in CF mice, and that SCV mutants readily emerged over time in this background. The phenomenon of SCV morphotypes arising in hypermutable *P. aeruginosa* backgrounds has been independently observed for another  $\Delta$ *mutS* mutant strain,<sup>80</sup> which was also shown to produce a large number of mutations in the *lasR* quorum sensing locus.<sup>36</sup> The work of Mena et al<sup>35</sup> strongly suggests that mismatch repair mutants are selected in chronic *P. aeruginosa* lungs due to their increased persistence. As a possible explanation for this, hypermutable strains have been linked to the RsmA signaling network in *Erwinia carotovora*,<sup>81</sup> placing RsmA downstream of *mutS* and suggesting that the persistence of these strains may result (at least in part) from reduced levels of RsmA (Figure 5). Another possibility is simply that the increased rate of mutation in these strains facilitates the emergence of mutations in the Gac/Rsm or cdG signaling pathways. Genetic adaptation to the CF lung has been shown to be catalyzed by the initial acquisition of mismatch repair mutations, with subsequent mutations arising much more rapidly than in nonmutator lines.<sup>82</sup> Mutants in the cdG signaling genes *fleQ* and *wspF* were identified in this study, although the isolate library used was the same as described in the earlier work of Smith et al,<sup>6</sup> where the *wspF* and *fleQ* mutations were initially identified.

Large-scale DNA inversions have also been associated with the generation of morphological diversity, and hence potentially SCV generation, in the lung environment. In a study of CF lung isolates, Römmling et al<sup>83</sup> showed that 50% of tested *P. aeruginosa* genomes contained large chromosomal inversions. These inversions were exclusively detected in CF lung isolates, suggesting that they are selected during adaptation to chronic lung infection.<sup>83</sup> Chromosomal inversions have been linked to the mobile element IS*6100* and are proposed to represent a source of phenotypic variation,<sup>84</sup> similar to that emerging from the loss of mismatch repair described above.<sup>80</sup>

Furthermore, reversible genomic inversion has been shown to induce an SCV phenotype in *S. aureus*.<sup>85</sup>

Recently, the advent of next-generation sequencing has enabled the molecular basis of SCV formation to be examined much more closely. The complete genome sequences of at least two clinically-evolved SCVs have been published, allowing candidate causal mutations for generation of the SCV phenotype to be investigated. For SCV20265,<sup>86</sup> the mutational routes to SCV are known,<sup>42</sup> while for the urethral catheter SCV MH27,<sup>87</sup> the underlying genetics of the SCV phenotype has yet to be established. As sequencing technology continues to improve and becomes ever more accessible, it is likely that the coming years will see a much more complete examination of the mutational and regulatory routes to SCV generation in the CF lung environment.

## Summary

*P. aeruginosa* SCVs are frequently isolated from the lungs of CF patients. The appearance of these distinctive, small, autoaggregative colonies strongly correlates with both deteriorating lung function and associated poor clinical prognosis. Whether this connection is causal or simply correlative is not yet clear. Nonetheless, the importance of the SCV phenotype in persistence of *P. aeruginosa* infection is beyond doubt, with SCVs conferring both increased antibiotic tolerance and resistance to immune phagocytosis. Hopefully, an increased awareness of the importance of SCVs in chronic CF infection, alongside recent improvements in both diagnostic and analytical tools, will allow us to make headway in the treatment of these unusual morphotypes.

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## Disclosure

The author reports no conflicts of interest in this work.

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