Genomic and transcriptomic characterization of *Pseudomonas aeruginosa* small colony variants derived from a chronic infection model

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**Abstract**

Phenotypic change is a hallmark of bacterial adaptation during chronic infection. In the case of chronic *Pseudomonas aeruginosa* lung infection in patients with cystic fibrosis, well-characterized phenotypic variants include mucoid and small colony variants (SCVs). It has previously been shown that SCVs can be reproducibly isolated from the murine lung following the establishment of chronic infection with mucoid *P. aeruginosa* strain NH57388A. Using a combination of single-molecule real-time (PacBio) and Illumina sequencing we identify a large genomic inversion in the SCV through recombination between homologous regions of two rRNA operons and an associated truncation of one of the 16S rRNA genes and suggest this may be the genetic switch for conversion to the SCV phenotype. This phenotypic conversion is associated with large-scale transcriptional changes distributed throughout the genome. This global rewiring of the cellular transcriptomic output results in changes to normally differentially regulated genes that modulate resistance to oxidative stress, central metabolism and virulence. These changes are of clinical relevance because the appearance of SCVs during chronic infection is associated with declining lung function.

**DATA SUMMARY**

All genome sequences are deposited in NCBI GenBank under accession numbers CP013477, CP013478 and CP013479. Transcriptome data are deposited at the EMBL-EBI ENA database under study number PRJEB12456.

**INTRODUCTION**

Phenotypic variation is a hallmark of adaptation to the host during chronic bacterial infection. There is considerable interest in slow-growing subpopulations of bacteria, termed small colony variants (SCVs), due to their association with persistent infections [1, 2]. The SCV variant is common to diverse bacteria and is characterized by phenotypes including reduced growth, increased biofilm production [3], antibiotic resistance and hyperpiliation. SCVs have been described for a wide range of bacterial genera and species including *Staphylococcus aureus* [4, 5], *Staphylococcus epidermidis* [6], *Streptococcus* sp. [7, 8], *Enterococcus* [9], *Listeria* [10], *Burkholderia* [11], *Salmonella* [12], *Brucella* [13], *Lactobacillus*, *Serratia* and *Neisseria* [14]. In the case of *Pseudomonas aeruginosa*, SCVs are commonly associated with chronic infection of the lung in patients with cystic fibrosis (CF) [15, 16].

*P. aeruginosa* is the major proven cause of mortality in patients with CF and chronic infection leads to a progressive decline in pulmonary function and inevitably respiratory failure [17–19]. Despite intensive anti-pseudomonal chemotherapy greatly improving the prognosis for CF patients [20], the current median age at death for CF patients is around 30 years in developed countries [21]. The frequent failure of antibiotic therapy and host defences to eradicate *P. aeruginosa* from the CF lung is thought to be largely due to the increased antibiotic tolerance when growing in the biofilm state and the appearance of mucoid phenotypic variants that are a hallmark of adaptation in the chronically infected lung. A further complicating factor is the appearance of highly adherent SCVs that are adept at biofilm formation [15, 22, 23]. *P. aeruginosa* SCVs may
display high intracellular levels of c-di-GMP [23–27], enhanced biofilm formation, high fimbrial expression, repression of flagellar genes, resistance to phagocytosis and enhanced antibiotic resistance. Most importantly, the appearance of SCVs in the CF lung correlates with poor clinical outcome [11, 28–31].

There are a range of genetic changes that have been shown to be responsible for the phenotypic switch to the SCV phenotype in P. aeruginosa, including mutations in the Wsp system and yfbBNR operon that form part of the c-di-GMP regulatory system in P. aeruginosa [26, 32–34]. However, identification of the major clinically relevant pathways of conversion to the SCV phenotype is complicated by the unstable phenotype displayed by many SCVs with reversion to a normal colony phenotype frequently observed, preventing successful comparative genetic studies on clinical SCVs and their closely related parent strains. In S. aureus, which also forms clinically relevant SCVs, recent work has shown that a reversible large-scale chromosomal inversion is the genetic basis of the switch between a normal colony and SCV isolated from the same patient [35]. In addition, S. aureus SCVs, which are commonly isolated from the CF lung, are highly resistant to oxidative stress, suggesting that conversion to the SCV phenotype may be an adaptation to the environment in chronically inflamed host tissue [36].

In the present study, we have attempted to determine the genetic basis of phenotypic conversion from the mucoid to the SCV phenotype for SCVs isolated from the chronic lung infection model described by Bayes et al. [37]. For two SCVs isolated from this work, we have shown through a combination of single-molecule real-time (SMRT; Pacific Biosciences) and Illumina sequencing that a large and stable chromosomal inversion and associated truncation of a 16S rRNA gene accompanies conversion to the SCV phenotype. The phenotypic switch is characterized by transcriptional changes to a large number of genes that most notably include downregulation of several genes encoding metabolic enzymes, DNA repair proteins and heat shock proteins and upregulation of genes encoding proteins involved in the response to oxidative stress. The absence of other obvious genetic changes suggests that this chromosomal inversion may be the genetic basis of conversion to the SCV phenotype.

RESULTS

P. aeruginosa SCVs are commonly isolated from patients with CF and have been isolated in vitro as well as from experimental infection models following aminoglycoside treatment [16, 28]. Bayes et al. describe the isolation and partial characterization of SCVs isolated from a chronic murine P. aeruginosa lung infection model [37]. In this model, animals were inoculated with P. aeruginosa strain NH57388A (NHMuc), a mucoid clinical isolate, embedded in agar beads. NHMuc has a known mutation in the gene encoding the anti-sigma factor MucA, which results in alginate overproduction [38, 39]. Recovered bacteria from lung homogenate samples display two distinct colony morphologies: typical large mucoid colonies identical in morphology to the inoculating strain and SCVs. Mucoid colonies were evident after 24 h of growth on agar plates at 37 °C with SCVs visible only after 48 h of growth on agar plates [37].

To understand the genetic basis of this phenotypic change, we initially performed Illumina HiSeq whole-genome sequencing and genomic comparison between NHMuc and two separate SCVs (SCVJan and SCVFeb) isolated from independent in vivo experiments. However, despite their gross phenotypic differences, this analysis failed to identify any genetic differences between the SCVs and the parent strain.

Next, we used the ultra-long reads produced by SMRT PacBio sequencing to attempt to identify any large-scale genome rearrangements that could drive conversion to the SCV phenotype. Using this technique we identified a large-scale genomic inversion accompanying conversion from the mucoid to SCV phenotype in both SCVJan and SCVFeb. Closer inspection of the genome sequence identified the start and end points of the inversion, which for both SCVJan and SCVFeb begins at the first RNA operon (0.72 Mbp) and ends at the third RNA operon (5.21 Mbp). Exact chromosomal breakpoints were identified in the corresponding 16S rRNA genes by performing a MAUVE breakpoint analysis (Fig. 1). Furthermore, genome analysis revealed a 250 bp shortened 16S rRNA gene (16S1t) in both SCV strains, which is reflected in the reduced genome sizes of the SCVs (SCVJan 6213 026 bp, SCVFeb 6213 029 bp; Fig. 2a) compared to the parent strain, NHmuc (6213 276 bp; Fig. 2b). There were no further differences in the number of protein coding genes (5619), rRNAs (12) or tRNAs (57) between SCVs and the parent strain. No SNPs could be identified in protein coding genes. A similar genome inversion was not identified (using a PCR-based

IMPACT STATEMENT

Chronic lung infection with Pseudomonas aeruginosa is the major proven cause of mortality in patients with cystic fibrosis. This is despite the use of aggressive antibiotic therapy, which although effective in managing infection, frequently fails to eradicate P. aeruginosa. Adaptation to the lung is associated with the appearance of small colony variants (SCVs), which are highly adherent and adept at forming biofilms. Here we show that SCVs isolated from the murine lung during chronic infection, and in the absence of antibiotic therapy, show transcriptional changes that enhance the oxidative stress response and increase virulence. Sequencing of the genomes of the SCVs and parent strain shows that the SCVs contain a large genomic inversion relative to the parent strain.
strategy) in a mucoid strain (NHMucJan) that was phenotypically identical to the parent strain (NHMuc) and isolated from the same chronic infection model as SCVJan (Fig. S1, available in the online version of this article). Interestingly, comparison of the SCVJan and SCVFeb genomes with that of an SCV (SCV20265) isolated from a CF patient [40, 41], which has recently been sequenced by PacBio sequencing, revealed an almost identical chromosomal inversion. However, in SCV20265 the inversion was not accompanied by truncation of the 16S rRNA gene in the third rRNA operon (Fig. 1).

Transcriptional and phenotypic changes on conversion to the SCV phenotype

To determine the transcriptional changes associated with conversion to the SCV phenotype, we performed RNA sequencing (RNA-Seq) analysis of the parent strain, NHMuc, and two SCV strains grown in Lysogeny broth (LB). Initial analysis showed that SCVJan and SCVFeb have highly similar gene expression profiles that are distinct from that of NHMuc. RNA-Seq data for all strains were collected in triplicate and data for SCVJan and SCVFeb were combined to compare with NHMuc. Relative

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**Fig. 1.** A common large-scale chromosomal inversion in three *P. aeruginosa* strains is the genetic basis for conversion to the SCV phenotype. From top to bottom strains NHMuC, SCVJan, SCVFeb and SCV20265 are displayed. Dashed lines indicate the inversion breakpoints present in the 16S rRNA genes. An inversion with highly similar breakpoints is present in the genome of strain SCV20265, an SCV isolated from a patient with CF. Within strains SCVJan and SCVFeb, a unique truncated version of the 16S rRNA gene (16Sₚ) could be resolved, which could not be detected in strain SCV20265.
to NHMuc, 190 genes showed >2-fold upregulation and 364 genes showed >2-fold downregulation in SCVJan/SCVFeb (Table S1). Interestingly, the transcriptional changes associated with genomic inversion and that drive conversion to the SCV phenotype are not restricted to genes close to or within the inversion breakpoints, with major upregulated and downregulated genes distributed relatively evenly throughout the genome (Fig. 3).

Major functional classes of genes downregulated in SCVJan/SCVFeb include those involved in energy metabolism, amino acid and protein biosynthesis, DNA replication and recombination, and cell wall/lipopolysaccharide/capsule biosynthesis, which together are consistent with the slow growth rate observed for SCVs. Notably, genes encoding heat shock proteins and other molecular chaperones (IbpA, GrpE, HtpG, ClpB, DnaK, GroES, DnaJ and ClpX) are highly represented among the most strongly downregulated genes in the SCVs (Table 1). Conversely, genes that function in the response to oxidative stress and those that encode secreted virulence factors are largely upregulated in SCVJan/SCVFeb. Indeed, five of the ten most highly upregulated genes in SCVJan/SCVFeb are those associated with the response to oxidative stress (Table 2). Highly upregulated oxidative stress genes include katA [41], which encodes the major catalase of P. aeruginosa, ahpB, ahpC and ahpF [42, 43], which encode subunits of alkyl hydroperoxide reductase, and trxB2, which encodes thioredoxin reductase 2 [44, 45]. Consistent with the observed transcriptional changes, catalase activity was strongly increased in SCVJan relative to NHMuc (Fig. 4a).

Genes encoding a number of secreted virulence factors such as the proteases LasA, LasB and AprA, the fructose-binding lectin LecB [46] and the chitin binding protein CbpD and chitinase ChiC [47] were also highly upregulated. Similarly, genes encoding hydrogen cyanide synthase and a number of enzymes that function in phenazine biosynthesis are also upregulated (Table S1) [25, 48–51]. Phenazines have previously been shown to enhance killing of Caenorhabditis elegans by P. aeruginosa [52]. The apparent increase in the production of virulence factors by SCVJan/SCVFeb relative to NHMuc suggests increased virulence of the SCV. To directly test this we used an infection model based on infection of the murine macrophage cell line J774A.1. Cell death of J774A.1 through lactate
dehydrogenase (LDH) release was measured 4 and 10 h after infection with NHMuc and SCVJan. At 4 h, levels of LDH release were similar for NHMuc and SCVJan, whereas at 10 h LDH release was significantly increased for SCVJan (96 vs 38%, \( P < 0.0001 \); Fig. 4b). To determine if the increased virulence of the SCV observed against a murine cell line translated to increased virulence in an animal model of infection, we used an invertebrate model of infection using the larva of the wax moth Galleria mellonella. Similar to the macrophage infection assay, SCVJan displayed increased virulence in the G. mellonella infection model. Mortality of larvae was measured 24, 48 and 72 h after infection. No significant differences in mortality were detected at 24 or 48 h, whereas at 72 h mortality was 86 and 63% \( (P < 0.04) \) for SCVJan- and NHMuc-infected larvae, respectively (Fig. 4c). Data from both infection models indicate that SCVJan shows increased virulence, relative to NHMuc, which is consistent with the phenotype of SCVs obtained from the human host [29, 53].

**DISCUSSION**

In this work we show that P. aeruginosa SCVs isolated from a chronic murine lung infection model display a general upregulation of virulence-associated genes, relative to the mucoid parent strain, and increased virulence, which may begin to explain the link between the appearance of SCVs in chronic lung infection and the associated decline in lung function [54]. In addition, the immediate upregulation of genes that mediate the response to oxidative stress suggests why the isolated SCVs are rapidly selected for in a chronic infection model in which the host immune system is strongly activated.

A key strength of our study was the availability of both the parent strain used to establish infection in a chronic infection model and the derived SCVs that evolved during infection. This allowed for a meaningful comparative genetic analysis to be performed to probe the genetic changes that occurred on conversion to the SCV phenotype. Surprisingly, SNPs and short insertions and deletions (indels) were not identified in the SCV genome by Illumina sequencing, and SMRT sequencing was subsequently used to show that the two sequenced SCVs carried a large genomic inversion within 16S rRNA genes. Interestingly, the transcriptional changes associated with genomic inversion are not restricted to genes close to or within the inversion breakpoints, but distributed relatively evenly throughout the genome. Instead the major changes in gene expression are largely restricted to specific functional classes of genes including those that mediate the response to oxidative stress, virulence, DNA repair and recombination, the chaperone network and metabolism. This global rewiring of the cellular transcriptional output results in concerted transcriptional changes to these normally differentially regulated genes. However, the mechanisms that underlie these transcriptional changes are not clear. A study in *Escherichia coli* suggested that positional effects on gene expression may be due to local differences in chromosomal structuring and organization, with DNA gyrase playing an important role at certain high-activity locations [55]. Further studies will be needed to clarify such positional effects on gene expression in the SCV studied here.

Other large-scale genome rearrangements including large chromosomal inversions have previously been described in *P. aeruginosa*, but these were not associated with conversion to the SCV phenotype [56]. A reversible genomic
A possible explanation for this observation is that a number of genes encoding proteins involved in DNA repair and recombination, including RecA, are downregulated in the SCV relative to the parent strain (Table 1).

In conclusion, we have shown that a *P. aeruginosa* SCV that originated in the lungs of an animal with chronic colonization may result from a large chromosomal inversion and associated truncation of an rRNA gene. Conversion to the SCV phenotype was associated with large-scale transcriptional changes and increased virulence.

**METHODS**

**Genome assembly and annotation**

Purified bacterial genomic DNA was prepared for sequencing on an Illumina HiSeq using Qiagen DNeasy Blood and Tissue Kit as per the manufacturer’s protocol. Sequencing and initial bioinformatics were performed in the Centre for Genomic Research, University of Liverpool. Sequencing reads were mapped to the corresponding reference genome (annotated NH strain). SMRTbell template libraries were prepared according to the instructions from Pacific Biosciences, following the Procedure and Checklist Greater than 10 kb Template Preparation and Sequencing. Briefly, for preparation of 10 kb libraries, ~10 µg of genomic DNA isolated from SCVJan, SCVFeb and NHmuc was sheared using g-tubes from Covaris according to the manufacturer’s instructions. In total, 5–10 µg of sheared genomic DNA was end-repaired and ligated overnight to hairpin adapters applying components from the DNA/Polymerase Binding Kit P4 from Pacific Biosciences. Reactions were carried out according to the manufacturer’s instructions. SMRTbell template was treated with exonuclease for removal of incompletely formed reaction products. Conditions for annealing of sequencing primers and binding of polymerase to purified SMRTbell template were assessed with the Calculator in RS Remote (Pacific Biosciences). SMRT sequencing was carried out on the PacBio RSII (Pacific Biosciences) taking one 180 min movie for each SMRT cell. In total six, six and five SMRT cells were run respectively. Data from each SMRT cell were assembled independently using the ‘RS_HGAP_Assembly.3’ protocol included in SMRTPortal version 2.3.0 using default parameters. Each assembly revealed the fully resolved chromosome in one single contig. Each chromosome was circularized independently; in particular, artificial redundancies at the ends of the contigs were removed and all chromosomes were additionally adjusted to *dnaA* as the first gene. The validity of each assembly was checked using the ‘RS_Bridge-mapper.1’ protocol. For the purpose of this study, it has been confirmed for each of the (repetitive) rRNA operons that enough uniquely mapping long read exists spanning the whole repeat structure. Finally, each genome was error-corrected by a mapping of Illumina reads (paired-end reads, 100 bp) onto finished genomes using Burrows–Wheeler Transform (BWA) [57] with subsequent variant calling using VarScan [58]. A consensus concordance of QV60 could be confirmed for all of the three genomes. Finally, all genomes

![Phenotypic characterization of SCVJan.](image_url)

**Fig. 4.** Phenotypic characterization of SCVJan. (a) Catalase activity assay demonstrating a marked increase in catalase activity in the SCV as compared to the NH parent strain. (b) Percentage lactate dehydrogenase (LDH) released from a macrophage cell line with comparison between NHMuc and SCVJan over a 4 and 10 h time period. (c) *Galleria mellonella* larvae survival over time when infected with the NHMuc and SCVJan strains monitored over 72 h.
Bacterial suspensions were grown to early stationary phase to

Transcriptome analysis

reads only at that genome position (data not shown). against the final chromosome showing uniquely mapped Bio assembly as well as BWA mapping of Illumina reads gene for strains SCVJan and SCVFeb was confirmed by Pac-

were annotated using Prokka 1.8 [59]. All genome sequences were deposited in NCBI GenBank under accession numbers CP013477, CP013478 and CP013479. Illumina short read data have been deposited at the EMBL-EBI ENA database under study number PRJEB12456. The shortened 16S rRNA gene for strains SCVJan and SCVFeb was confirmed by Pac-Bio assembly as well as BWA mapping of Illumina reads against the final chromosome showing uniquely mapped reads only at that genome position (data not shown). Genome maps were created using DNAplotter65 [60].

Table 1. Highly downregulated genes in SCVs relative to NHMuc

| Gene ID       | Fold change* | P†       | Protein description                  | PA01 gene ID |
|---------------|--------------|----------|--------------------------------------|--------------|
| NHmuc_01025   | -75          | 2.6 × 10^{-11} | Putative acetyltransferase           | PA4364       |
| mexC          | -53          | 1.1 × 10^{-11} | RND multidrug efflux fusion protein MexC | PA4599       |
| lbpA          | -31          | 1.1 × 10^{-7}  | Heat-shock protein LbpA              | PA3126       |
| algD          | -30          | 1.0 × 10^{-9}  | GDP-mannose 6-dehydrogenase AlgD     | PA3540       |
| NHmuc_05385   | -29          | 8.5 × 10^{-8}  | 17 kDa surface antigen               | PA5182       |
| NHmuc_04138   | -29          | 1.3 × 10^{-10} | Periplasmic metal-binding protein    | PA3520       |
| grpE          | -25          | 1.2 × 10^{-7}  | Heat shock protein GrpE              | PA4762       |
| htpG          | -23          | 3.6 × 10^{-7}  | Heat shock protein 90                | PA1596       |
| NHmuc_0100    | -23          | 1.8 × 10^{-5}  | Hypothetical, unclassified, unknown  |              |
| clpB          | -21          | 6.5 × 10^{-6}  | ClpB protein                         | PA4542       |
| nfxB          | -21          | 3.7 × 10^{-7}  | Transcriptional regulator NfxB       | PA4600       |
| fsxA          | -18          | 7.9 × 10^{-8}  | FxsA protein                         | PA4387       |
| NHmuc_04950   | -18          | 3.3 × 10^{-6}  | Molecular chaperone DnaK             | PA4761       |
| NHmuc_05744   | -17          | 5.8 × 10^{-5}  | Putative lipoprotein                 | PA5526       |
| dapB          | -17          | 2.8 × 10^{-6}  | Dihydropicolinate reductase          | PA4759       |
| hslV          | -17          | 4.1 × 10^{-7}  | ATP-dependent protease subunit       | PA5053       |
| NHmuc_01173   | -16          | 4.7 × 10^{-6}  | Hypothetical protein                 | PA2756       |
| NHmuc_01024   | -16          | 6.1 × 10^{-7}  | Putative transporter                 | PA4365       |
| NHmuc_04180   | -16          | 3.3 × 10^{-6}  | Recombinase A                       | PA3617       |
| mexD          | -15          | 9.5 × 10^{-7}  | RND multidrug efflux transporter MexD | PA4598       |
| NHmuc_04776   | -15          | 6.1 × 10^{-6}  | PAS/PAC sensor signal transduction histidine kinase | PA4197 |
| rsmA          | -15          | 7.5 × 10^{-4}  | Carbon storage regulator             | PA0905       |
| NHmuc_01174   | -14          | 3.3 × 10^{-7}  | Hypothetical protein                 | PA0737       |
| NHmuc_01594   | -13          | 2.1 × 10^{-5}  | Putative oxidoreductase              | PA1137       |
| mucA          | -13          | 7.4 × 10^{-5}  | Anti-sigma factor MucA               | PA0763       |
| NHmuc_03262   | -12          | 2.5 × 10^{-9}  | Hypothetical protein                 | PA3505       |
| NHmuc_04386   | -12          | 6.8 × 10^{-5}  | Surface antigen                      | PA3819       |
| amrZ          | -12          | 3.0 × 10^{-5}  | Alginate and motility regulator Z    | PA3385       |
| glmA          | -12          | 5.8 × 10^{-6}  | Glutamine synthetase                 | PA5119       |
| groES         | -12          | 3.9 × 10^{-5}  | Co-chaperonin GroES                  | PA4386       |
| dnaJ          | -12          | 7.5 × 10^{-5}  | Chaperone protein DnaJ               | PA4760       |
| algU          | -11          | 1.8 × 10^{-4}  | RNA polymerase sigma factor AlgU     | PA0762       |
| clpX          | -11          | 4.5 × 10^{-4}  | ATP-dependent protease subunit ClpX  | PA1802       |
| NHmuc_04521   | -11          | 1.9 × 10^{-6}  | Periplasmic ligand-binding sensor     | PA3952       |

*The magnitude of gene expression (fold change) was determined by comparing transcription in three replicates of NH with that in three replicates each of the two SCV strains.

†P-values were assessed by performing an EDGE test using CLC software.

were isolated using the Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen) as per the manufacturer’s instructions. an OD$_{600}$ of 1.8 in LB at 37 °C in a shaking incubator. Then, 2 ml of each suspension was pelleted at 12 000 g for 10 min. RNA was extracted from samples using a bead beating/chloroform extraction method as previously described [61]. The samples were digested with DNase I for 1 h. Bacterial RNA was enriched using MICROBEnrich (Life Technologies) as per the suggested protocol. rRNA was depleted using Ribo-Zero Magnetic Gold Kit (Epidemiology; Epicentre) as per the manufacturer’s protocol. The precipitated sample was resuspended in 20 µl of RNase-free water. The concentration of RNA was initially determined using Nanodrop followed by an Agilent Bioanalyzer. cDNA was generated by using the methods from the Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen) as per the manufacturer’s instructions.
Transcriptome analysis was performed using CLC workbench version 7.0 and significantly upregulated and downregulated genes in SCVJan/SCVFeb versus NHMuc were identified using the CLC software package. Transcriptome data were deposited at the EMBL-EBI ENA database under study number PRJEB12456.

## PCR

Genomic DNA was extracted from 1.5 ml of bacterial culture using the GenElute Bacterial Genomic DNA Kit (Sigma). Extraction was performed following the manufacturer’s recommendations and DNA was eluted into 100 µl of Elute Solution. PCR detection of the inversion was performed with KAPA polymerase (KAPA Long-range HotStart PCR Kit; KAPA Biosystems) following the manufacturer’s protocol. Two sets of primers were used: birA-F/yedZ-R and birA-F/tyrZ-R (birA-F: CTCACCGAGTGGAAATC, yedZ-R: TGAGCGTTACTGGTATCCATGG and tyrZ-R: CCATACCGTGTATTATAAAGC) with the genomic DNAs of the mucoid and SCV strains recovered from animals amplifying a fragment of 6262 or 6960 bp, respectively.

### Galleria mellonella infection model

Larvae were stored on wood chips at 4℃. Overnight cultures of bacterial strains were grown in LB, diluted 1 : 100 in the same medium and grown to an OD 

### Table 2. Highly upregulated genes in SCVs relative to NHMuc

| Gene ID        | Fold change* | P^† | Protein description                                      |
|---------------|--------------|-----|---------------------------------------------------------|
| NHmuc_04720   | 62           | 3.2×10^{-16} | Hypothetical protein                                    |
| trxB2         | 55           | 4.0×10^{-16} | Thioredoxin reductase 2                                 |
| NHmuc_01300   | 35           | 3.7×10^{-15} | Putative alkyl hydroperoxide reductase                  |
| NHmuc_02350   | 28           | 1.4×10^{-26} | Putative acyl carrier protein                           |
| ahpF          | 28           | 8.4×10^{-13} | Alkyl hydroperoxide reductase subunit F                 |
| kata          | 23           | 7.1×10^{-10} | Catalase                                                |
| NHmuc_03862   | 20           | 6.9×10^{-21} | Putative ankyrin domain-containing protein              |
| NHmuc_03863   | 19           | 1.8×10^{-18} | Putative hydrolase                                     |
| NHmuc_00257   | 19           | 1.4×10^{-12} | Putative CBS domain protein                             |
| ahpC          | 18           | 9.0×10^{-8}  | Alkyl hydroperoxide reductase subunit C                 |
| chiC          | 17           | 1.5×10^{-9}  | Chitinase                                              |
| NHmuc_04185   | 17           | 6.7×10^{-6}  | RNA polymerase sigma factor RpoS                        |
| aprA          | 12           | 5.1×10^{-8}  | Alkaline metallocprotein                                |
| NHmuc_04924   | 11           | 3.2×10^{-16} | CdbD family protein                                    |
| NHmuc_04718   | 11           | 1.5×10^{-11} | Hypothetical protein                                   |
| NHmuc_04925   | 11           | 2.2×10^{-8}  | Transport-associated                                   |
| NHmuc_00127   | 10           | 5.9×10^{-7}  | Putative haemolysin                                    |
| Snr1          | 10           | 4.9×10^{-7}  | Cytochrome c Snr1                                      |
| lecB          | 9            | 5.9×10^{-8}  | Fucose-binding lectin PA-III                            |
| NHmuc_03413   | 8            | 9.1×10^{-15} | Phage terminase, small subunit                          |
| katB          | 8            | 9.1×10^{-6}  | Catalase                                               |
| NHmuc_04074   | 8            | 2.2×10^{-12} | Leucyl-tRNA synthetase                                 |
| phzG2_2       | 7            | 3.3×10^{-6}  | Pyridoxamine 5’-phosphate oxidase                       |
| NHmuc_03358   | 7            | 2.0×10^{-8}  | Putative protein associated inclusion bodies            |
| phzE_1        | 7            | 3.2×10^{-8}  | Phenazine biosynthesis protein PhzE                     |
| NHmuc_00055   | 7            | 6.1×10^{-7}  | Hypothetical protein                                   |
| NHmuc_01628   | 7            | 1.3×10^{-8}  | Hypothetical protein                                   |
| ctpD          | 7            | 4.1×10^{-6}  | Chitin-binding protein CtpD                             |
| NHmuc_00546   | 6            | 2.0×10^{-3}  | LysR transcriptional regulator                         |
| rhR           | 6            | 1.2×10^{-4}  | Transcriptional regulator RhR                          |
| gcdH          | 6            | 4.3×10^{-5}  | Glutaryl-CoA dehydrogenase                             |
| NHmuc_01422   | 6            | 4.1×10^{-4}  | Putative DNA-binding stress protein                     |
| NHmuc_04078   | 6            | 1.5×10^{-10} | Oxidoreductase probably involved in sulphite reduction |
| rsaL          | 6            | 1.2×10^{-2}  | Regulatory protein RsaL                                 |

*The magnitude of gene expression (fold change) was determined by comparing transcription in three replicates of NH with that in three replicates each of the two SCV strains.

†P-values were assessed by performing an EDGE test using CLC software.
OD$_{600}$ of 0.1. Serial 10-fold dilutions were made in PBS. Five-microlitre aliquots of the serial dilutions were injected using a Hamilton syringe into G. mellonella larvae, via the hindmost left proleg as previously described [64]. Ten larvae were injected per dilution for each Pseudomonas strain tested. Larvae were incubated in 10 cm plates at 37°C and the number of dead larvae was scored 1–4 days after infection. For each strain, data from three independent experiments were combined. Larvae were considered dead when they displayed no movement in response to touch. A negative control was used in each experiment to monitor the killing due to physical injury or infection by pathogenic contaminants. Time to death was monitored every 24 h after infection. In any instance where more than one control larvae died in any given experiment, the data from infected larvae were not used.

**LDH release/cytotoxicity assay**

To investigate the effect of the P. aeruginosa strains on macrophages, we infected the J774A.1 cells with NH and SCV. Bacteria were grown for 17 h to stationary phase in LB at 37°C. Immediately prior to infection, the bacteria were diluted to exponential growth phase with culture medium lacking phenol red and the concentration was determined by measuring the optical density at 600 nm. Cells were grown, washed and infected as previously documented [65]. Cells were infected with test organisms and incubated for 4 and 10 h. LDH release was determined using the Cytotox 96 cytotoxicity assay kit (Promega) as per the manufacturer’s protocol.

**Catalase activity assay**

Overnight cultures of bacterial strains were grown in LB, diluted 1:100 in the same medium and grown to an OD$_{600}$ of 0.4. Catalase standards were prepared as per the manufacturer’s protocol using the OxiSelect Catalase Activity Assay Kit, Colorimetric (Cell Biolabs). In total, 20 µl of each serial dilution of overnight culture was added to three wells in a 96-well plate to allow for average readings for each sample. Plate absorbance was read at 520 nm using a FLUOstar Optima plate reader (BMG UK).

**Accessibility of biological resources**

SCVs used in this study have been deposited at DSMZ under DSM 100776–100778.

**Funding information**

This work was funded by an MRC SCP3 fellowship grant G1000419, Welcome Trust grant 201505/Z/16/2, and by grant 8000-105-3 of the German Federal Ministry of Science and Education through the German Centre of Infection Research (DZIF) to J. O. We thank Simone Severitt and Nicole Mrotzek at the Leibniz Institute DSMZ, Germany, for excellent technical assistance.

**Author Contributions**

S. I., D. W., B. B. and J. O. conceived and designed the experiments and analysis. S. I., B. B., C. S. and A. S. performed the experiments. S. I., B. B., J. P. R. C. and A. J. R. analysed the data. T. J. E. and H. K. B. supplied novel reagents. D. W. and S. I. wrote the manuscript.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Data Bibliography**

Eckweiler D, Bunk B, Sprör C, Overmann J, Häussler S. Complete Genome Sequence of Small-Colony Variant SCV20265. DDBJ/EMBL/GenBank no. CP.

**References**

1. Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P et al. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Microbiol 2006; 4:295–305.
2. Grant SS, Hung DT. Persistent bacterial infections, antibiotic tolerance and the oxidative stress response. Virulence 2013;4:273–283.
3. Déziel E, Comeau Y, Villermur R. Initiation of biofilm formation by Pseudomonas aeruginosa 57RP correlates with emergence of hyperpiliated and highly phenotypic variants deficient in swimming, swarming, and twitching motilities. J Bacteriol 2001; 183:1195–1204.
4. Mirani ZA, Aziz M, Khan SI. Small colony variants have a major role in stability and persistence of Staphylococcus aureus biofilms. J Antibiot 2015;68:98–105.
5. Brouillet E, Martinez A, Boyll BJ, Allen NE, Malouin F. Persistence of a Staphylococcus aureus small-colony variant under antibiotic pressure in vivo. FEMS Immunol Med Microbiol 2004;41:35–41.
6. Maduka-Ezeh AN, Greenwood-Quaintance KE, Karau MJ, Berbci EF, Osmon DR et al. Antimicrobial susceptibility and biofilm formation of Staphylococcus epidermidis small colony variants associated with prosthetic joint infection. Diagn Microbiol Infect Dis 2012;74:224–229.
7. Allegrucci M, Sauer K. Formation of Streptococcus pneumoniae non-phase-variable colony variants is due to increased mutation frequency present under biofilm growth conditions. J Bacteriol 2008;190:6330–6339.
8. Zbinden A, Quiblier C, Hernandez D, Herzog K, Bodler P et al. Characterization of Streptococcus titurginus small-colony variants causing prosthetic joint infection by comparative whole-genome analyses. J Clin Microbiol 2014;52:467–474.
9. Wellinghamen N, Chatterjee I, Berger A, Niederfurth A, Proctor RA et al. Characterization of clinical Enterococcus faecalis small-colony variants. J Clin Microbiol 2009;47:2802–2811.
10. Rea R, Hill C, Gahan GG. Listeria monocytogenes PerR mutants display a small-colony phenotype, increased sensitivity to hydrogen peroxide, and significantly reduced murine virulence. Appl Environ Microbiol 2005;71:8314–8322.
11. Pinto-de-Oliveira A, Coutinho CP, Ramos CG, Sousa SA, de Carvalho CCCR et al. 109 the Burkholderia cepacia small colony variants (SCV) are a more pathogenic bacterial form that may facilitate persistent respiratory infections in CF patients. J Cyst Fibros 2013;12:576.
12. Cano DA, Pucciarelli MG, Martinez-Moya M, Casadesús J, Garcia-del Portillo F. Selection of small-colony variants of Salmonella enterica serovar typhimurium in nonphagocytic eucaryotic cells. Infect Immun 2003;71:3690–3698.
13. Jacob J, Hort GM, Overhoff P, Mielke ME. In vitro and in vivo characterization of smooth small colony variants of Brucella abortus S19. Microbes Infect 2006;8:363–371.
14. Morton HE, Shoemaker J. The identification of neisseria gonorrhoeae by means of bacterial variation and the detection of small colony forms in clinical material. J Bacteriol 1945;50:585–587.
15. Häussler S, Ziegler I, Löttel A, von Götz F, Rohde M et al. Highly adherent small-colony variants of Pseudomonas aeruginosa in cystic fibrosis lung infection. J Med Microbiol 2003;52:295–301.
31. Deretic V, Govan JR, Konyecsni WM, Martin DW. Mucoid Pseudomonas aeruginosa in cystic fibrosis: mutations in the muc loci affect transcription of the algR and algD genes in response to environmental stimuli. Mol Microbiol 1990;4:189–196.

32. Hickman JW, Tifrea DF, Harwood CS. A chemosensory system that regulates biofilm formation through modulation of cyclic di-guanosine monophosphate levels. Proc Natl Acad Sci USA 2005;102:14422–14427.

33. D’Argenio DA, Califfee MW, Rainey PB, Pesci EC. Autolysis and autoggregation in Pseudomonas aeruginosa colony morphology mutants. J Bacteriol 2002;184:6481–6489.

34. Cui L, Neoh HM, Iwamoto A, Hiramatsu K. Coordinated phenotype switching with large-scale chromosome flip-flop inversion observed in bacteria. Proc Natl Acad Sci USA 2012;109:E1647–E1656.

35. Painter KL, Strange E, Parkhill J, Bamford KB, Armstrong-James D et al. Staphylococcus aureus adapts to oxidative stress by producing H2O2-resistant small-colony variants via the SOS response. Infect Immun 2015;83:1830–1844.

36. Bayes HK, Ritchie N, Irvine S, Evans TJ. A murine model of early Pseudomonas aeruginosa lung disease with transition to chronic infection. Sci Rep 2016;6:35838.

37. Hoffmann N, Rasmussen TB, Jensen PØ, Stub C, Hentzer M et al. Novel mouse model of chronic Pseudomonas aeruginosa lung infection mimicking cystic fibrosis. Infect Immun 2005;73:2504–2514.

38. Boucher JC, Yu H, Mudd MH, Deretic V. Mucoid Pseudomonas aeruginosa in cystic fibrosis: characterization of muc mutations in clinical isolates and analysis of clearance in a mouse model of respiratory infection. Infect Immun 1997;65:3838–3846.

39. Eckweiler D, Bunk B, Spröer C, Overmann J, Häussler S. Complete genome sequence of highly adherent Pseudomonas aeruginosa small-colony variant SCV20265. Genome Announc 2014;2:e01232–13.

40. Asa JS, Heo YJ, Lee JK, Cho YH, KatA, the major catalase, is critical for osmoregulation and virulence in Pseudomonas aeruginosa PA14. Infect Immun 2005;73:4399–4403.

41. Ochsner UA, Vasil ML, Alsabbagh E, Parvatiyar K, Hassett DJ. Role of the Pseudomonas aeruginosa oxyR-recG operon in oxidative stress defense and DNA repair: OxyR-dependent regulation of katB-ankB, ahpB, and ahpC-ahpF. J Bacteriol 2000;182:4533–4544.

42. Vinckx T, Matthys S, Cornelis P. Loss of the oxidative stress regulator OxyR in Pseudomonas aeruginosa PA01 impairs growth under iron-limited conditions. FEMS Microbiol Lett 2008;288:258–265.

43. Chung JS, Noguera-Mazon V, Lancelin JM, Kim SK, Hirasawa M et al. Interaction domain on thioredoxin for Pseudomonas aeruginosa 5'-adenylylsulfate reductase. J Biol Chem 2009;284:31181–31189.

44. Hishinuma S, Ohtsu I, Fujimura M, Fukumori F. OxyR is involved in the expression of thioredoxin reductase TrxB in Pseudomonas putida. FEMS Microbiol Lett 2008;289:139–145.

45. Tielker D, Hacker S, Loris R, Strathmann M, Wingender J et al. Pseudomonas aeruginosa lectin LecB is located in the outer membrane and is involved in biofilm formation. Microbiology 2005;151:1313–1323.

46. Frederiksen RF, Paspaliari DK, Larsen T, Storgaard BG, Larsen MH et al. Bacterial chitinases and chitin-binding proteins as virulence factors. Microbiology 2013;159:833–847.

47. Mavrodi DV, Bonsall RF, Delaney SM, Soule MJ, Phillips G et al. Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from Pseudomonas aeruginosa PA01. J Bacteriol 2001;183:6454–6465.

48. Lau GW, Ran H, Kong F, Hassett DJ, Mavrodi D. Pseudomonas aeruginosa pyocyanin is critical for lung infection in mice. Infect Immun 2004;72:4275–4278.

49. Pessi G, Williams F, Hindle Z, Heurlier K, Holden MT et al. The global posttranscriptional regulator RsmA modulates production of virulence determinants and N-acylhomoserine lactones in Pseudomonas aeruginosa. J Bacteriol 2001;183:6676–6683.

50. Sánchez P, Linares JF, Ruiz-Diez B, Campanario E, Navas A et al. Fitness of in vitro selected Pseudomonas aeruginosa nalB and nfxB multidrug resistant mutants. J Antimicrob Chemother 2002;50:657–664.

51. Cezairiyan B, Vinayavekhin N, Grenfell-Lee D, Yuen GJ, Saghianian A et al. Identification of Pseudomonas aeruginosa phenazines that kill Caenorhabditis elegans. PLoS Pathog 2013;9:e1003101.
53. Sabra W, Haddad AM, Zeng AP. Comparative physiological study of the wild type and the small colony variant of Pseudomonas aeruginosa 20265 under controlled growth conditions. World J Microbiol Biotechnol 2014;30:1027–1036.

54. Evans TJ. Small colony variants of Pseudomonas aeruginosa in chronic bacterial infection of the lung in cystic fibrosis. Future Microbiol 2015;10:231–239.

55. Bryant JA, Sellars LE, Busby SJ, Lee DJ. Chromosome position effects on gene expression in Escherichia coli K-12. Nucleic Acids Res 2014;42:11383–11392.

56. Klockgether J, Munder A, Neugebauer J, Davenport CF, Stanke F et al. Genome diversity of Pseudomonas aeruginosa PA01 laboratory strains. J Bacteriol 2010;192:1113–1121.

57. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 2009;25:1754–1760.

58. Koboldt DC, Zhang Q, Larson DE, Shen D, McLellan MD et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Res 2012;22:568–576.

59. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics 2014;30:2068–2069.

60. Carver T, Thomson N, Bleasby A, Berriman M, Parkhill J. DNAPlotter: circular and linear interactive genome visualization. Bioinformatics 2009;25:119–120.

61. Lim YW, Schmieder R, Haynes M, Willner D, Furlan M et al. Metagenomics and metatranscriptomics: windows on CF-associated viral and microbial communities. J Cyst Fibros 2013;12:154–164.

62. Ramarao N, Nielsen-Leroux C, Lereclus D. The insect Galleria mellonella as a powerful infection model to investigate bacterial pathogenesis. J Vis Exp 2012:e4392.

63. Miyata S, Casey M, Frank DW, Ausubel FM, Drenkard E. Use of the Galleria mellonella caterpillar as a model host to study the role of the type iii secretion system in Pseudomonas aeruginosa pathogenesis. Infect Immn 2003;71:2404–2413.

64. Jander G, Rahme LG, Ausubel FM. Positive correlation between virulence of Pseudomonas aeruginosa mutants in mice and insects. J Bacteriol 2000;182:3843–3845.

65. Hauser AR, Engel JN. Pseudomonas aeruginosa induces type-iii-secretion-mediated apoptosis of macrophages and epithelial cells. Infect Immn 1999;67:5530–5537.