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

Sharon Irvine, Boyke Bunk, Hannah K. Bayes, Cathrin Spröer, James P. R. Connolly, Anne Six, Thomas J. Evans, Andrew J. Roe, Jörg Overmann and Daniel Walker

**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 yjB 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 N57388A (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 parent 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 rRNA operon (0.72 Mbp) and ends at the third rRNA 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 (16St) in both SCV strains, which is reflected in the reduced genome sizes of the SCVs (SCVJan 6.213.026 bp, SCVFeb 6.213.029 bp; Fig. 2a) compared to the parent strain, NHmuc (6.213.276 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 pheno-
typically identical to the parent strain (NHMuc) and iso-
lated from the same chronic infection model as SCVJan
(Fig. S1, available in the online version of this article). Inter-
estingly, 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 dis-
tinct 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
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 transcriptomic 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
inversion has also recently been shown to mediate the reversible conversion between normal colony and SCV phenotypes in *S. aureus* [35]. However, in the case of the SCVs isolated in our work, the SCV phenotype is stable and revertants to the parent phenotype were not observed.

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

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**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

Genome maps were created using DNAplotter65 [60]. Against the final chromosome showing uniquely mapped
gene for strains SCVJan and SCVFeb was confirmed by Pac-
data have been deposited at the EMBL-EBI ENA database
were annotated using Prokka 1.8 [59]. All genome sequences
were deposited in NCBI GenBank under accession numbers
were deposited in NCBI GenBank under accession numbers

Table 1. Highly downregulated genes in SCVs relative to NHMuc

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<th>Gene ID</th>
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<th>P†</th>
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<td>mexC</td>
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*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 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].

Transcriptome analysis

RNA isolation of the samples was performed in triplicate.
Bacterial suspensions were grown to early stationary phase to
an OD₆₀₀ 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/chlo-
roform 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. RNA was depleted using Ribó-
Zero Magnetic Gold Kit (Epidemiology; Epicentre) as per the
manufacturer’s protocol. The precipitated sample was resus-
pended in 20 µl of RNase-free water. The concentration of
RNA was initially determined using Nanodrop followed by an
Agilent Bioanalyser. cDNA was generated by using the
methods from the Superscript Double-Stranded cDNA Syn-
thesis 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 SCV Jan/SCV Feb 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: CTCACCGGAGTGGAATC, yedZ-R: TGAACGTACTGCATGTGTCACTCGG and tyrZ-R: CCATACCGTCATATTAAAGC) 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 °C. Overnight cultures of bacterial strains were grown in LB, diluted 1:100 in the same medium and grown to an OD600 of 0.3–0.4 as previously described [62, 63]. Cultures were centrifuged and pellets were washed twice and resuspended in 10 mM PBS to an

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<td>Putative acyl carrier protein</td>
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<td>rsaL</td>
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*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 morganisms, we infected the J774.A1 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**

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**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**

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