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1 ***Pseudomonas aeruginosa* strains from both clinical and environmental origins readily**
2 **adopt a stable small colony variant (SCV) phenotype resulting from single mutations in c-**
3 **di-GMP pathways**
4
5
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16 Running Head: Emergence of SCVs in *Pseudomonas aeruginosa*
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21 **ABSTRACT**

22 A subpopulation of Small Colony Variants (SCVs) is a frequently observed feature of
23 *Pseudomonas aeruginosa* isolated from colonized cystic fibrosis lungs. Since most SCVs have
24 until now been isolated from clinical samples, it remains unclear how widespread is the ability of
25 *P. aeruginosa* to develop this phenotype and the genetic mechanism(s) behind SCVs emergence
26 according to the origin of the isolate. In the present work, we investigated the ability of 22 *P.*
27 *aeruginosa* isolates from various environmental origins to, under laboratory culture conditions,
28 spontaneously adopt a SCV-like smaller alternative morphotype distinguishable from the
29 ancestral parent strain. We found that all the *P. aeruginosa* strains tested could adopt a SCV
30 phenotype, regardless of their origin. Whole genome sequencing of SCVs obtained from clinical
31 and environmental sources revealed single mutations exclusively in two distinct c-di-GMP
32 signaling pathways, Wsp and YfiBNR. We conclude that the ability to switch to a SCV
33 phenotype is a conserved feature of *P. aeruginosa* and results from the acquisition of a stable
34 genetic mutation, regardless of the origin of the strain.

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37 **IMPORTANCE**

38 *P. aeruginosa* is an opportunistic pathogen that thrives in many environments. It poses a
39 significant health concern, notably because this bacterium is the most prevalent pathogen found
40 in the lungs of people with cystic fibrosis. In infected hosts, its persistence is considered related
41 to the emergence of an alternative small colony variant (SCV) phenotype. By reporting the
42 distribution of *P. aeruginosa* SCVs in various non-clinical environments and the involvement of
43 c-di-GMP in SCV emergence from both clinical and environmental strains, this work contributes
44 to understanding a conserved adaptation mechanism used by *P. aeruginosa* to adapt readily in all
45 environments. Hindering this adaptation strategy could help control *P. aeruginosa* persistent
46 infection.

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48 **INTRODUCTION**

49 The high genomic and metabolic diversity of *Pseudomonas aeruginosa* allows this
50 bacterium to thrive in diverse environments, such as aquatic habitats, soil, food, and even built
51 environments, such as hospital premise plumbing systems (1-3). This opportunistic pathogen,
52 frequently identified as a causative agent of nosocomial infections, is a major cause of infections
53 in immunocompromised individuals. Notably, *P. aeruginosa* is the most prevalent pathogen
54 found in the lungs of people with cystic fibrosis (CF) (4-6).

55 *P. aeruginosa* expresses a broad range of virulence determinants that counteract the host
56 immunity and promote survival (7). One of these factors is the ability to form biofilms. These
57 organized communities largely contribute to evade host immunity and antimicrobial treatments.
58 For instance, the biofilm matrix delays penetration of antibiotics and host defense effectors (8-
59 10). *P. aeruginosa* typically persists in the lungs of CF individuals as a biofilm (11, 12).

60 The emergence of a subpopulation of Small Colony Variants (SCVs) is a frequently
61 observed feature of *P. aeruginosa* isolates from CF lungs biofilms (13, 14). SCVs are
62 characterized by circular opaque dwarf colonies with a diameter about three-time smaller than
63 wild-type (WT) colonies (14-17). Shortly after their first report, we proposed that SCVs are
64 phenotypic variants (18). Several studies suggest that phenotypic switching could be regulated
65 by a reversible adaptation mechanism: phase variation (18, 19), traditionally defined as a high-
66 frequency ON/OFF switch between phenotypes in a heritable and reversible manner (20-22).
67 Indeed, SCVs spontaneously revert to the WT-like morphotype (15, 16, 18, 23). Yet, recent
68 studies have reported stable genetic mutations in *P. aeruginosa* leading to SCV phenotype in *in*
69 *vitro* grown biofilms and animal model of PA14 infection (14, 24, 25). The SCV phenotype is
70 typically caused by mutations in genes involved in the metabolism of the intracellular second

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71 messenger c-di-GMP (14, 26). Among them, mutations in the Wsp (Wrinkly Spreader) pathway
72 are the most frequently reported (14, 24, 27). The Wsp pathway is a chemosensory system
73 resulting in activation of the diguanylate cyclase (DGC) WspR in response to surface sensing,
74 which regulates the c-di-GMP pool, along with other DGCs (synthesis of c-di-GMP) and
75 phosphodiesterases (PDE, degradation of c-di-GMP) in *P. aeruginosa* (28-30).

76 C-di-GMP is largely involved in regulation of the phenotypic properties associated with
77 SCVs, though binding to specific receptors. For instance, while an overproduction of
78 exopolysaccharides (EPS) (Pel and Psl) (14, 31) and a motility deficiency, notably flagellar, has
79 been described for SCVs (16, 18, 32), high c-di-GMP levels activate the expression of the *pel*
80 operon, leading to production of the EPS Pel, and repress flagellar motility (33-35). *P.*
81 *aeruginosa* SCVs exhibit several other specific properties such as cell surface hyperpiliation and
82 adherence to abiotic surfaces (16, 18, 36). These properties promote biofilm formation (37).
83 Additionally, SCVs exhibit autoaggregative properties (16, 36).

84 It is striking that SCVs have been mostly isolated from infected hosts, essentially CF
85 individuals; or by extension, from laboratory cultivation of strains sampled from infected hosts
86 (13). For instance, several studies have recovered SCVs from lung, sputum or deep throat swabs
87 of CF individuals (12, 16, 17, 38). CF is not the only pathology associated with the emergence of
88 *P. aeruginosa* SCVs. These variants have also been isolated from urine, feces, endotracheal
89 secretion and pleural effusion of patients suffering from meningioma, anoxic encephalopathy,
90 hepatocellular carcinoma, lung carcinoma or grave asphyxia neonatorum (39). In addition to having
91 been isolated from infected hosts, SCVs have also been generated under *in vivo* laboratory
92 conditions. For instance, SCVs have been obtained *in vivo* from *P. aeruginosa* strains during
93 infections in burn wound porcine models and murine models (24, 40). In the latter study, the authors

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94 have clearly showed that SCVs arisen in infection context are due stable genetic mutation in their
95 genomes (24).

96 Intriguingly, 20 years ago we reported one of the first identification of *P. aeruginosa* SCVs
97 that quickly emerged when a soil isolate was grown on a non-aqueous phase liquid, hexadecane,
98 as sole substrate (18). The SCV morphotype of strain 57RP predominates when biofilm growth
99 conditions are preferable and displays features shared with clinical SCVs: high adherence,
100 efficient biofilm formation, hyperpiliation and reduced motility (18).To our knowledge, this
101 study is the only one reporting SCVs for an environmental *P. aeruginosa* isolate. However, the
102 genetic cause leading to SCV emergence in the environmental context remains elusive. SCVs
103 generated *in vitro* from *P. aeruginosa* PAO1 and PA14 showed stable mutations, but these strains,
104 although prototypical, are still of clinical origin (25, 36).

105 Since most SCVs have until now been isolated from clinical samples, it remains unclear
106 how widespread is the ability of *P. aeruginosa* to develop this phenotype and the genetic
107 mechanism(s) behind SCVs emergence in regard to the origin of the isolate: are they exploiting
108 phase variation or selecting adaptive mutants? Here, we investigated the ability of *P. aeruginosa*
109 isolates from various environmental origins to spontaneously adopt, under laboratory culture
110 conditions, a SCV-like smaller colony morphotype readily distinguishable from their ancestral
111 parent. We tested 22 *P. aeruginosa* strains from four different categories of environments: soil,
112 food, hospital water systems and clinical; we found that all the *P. aeruginosa* strains have the
113 ability to adopt the SCV phenotype, regardless of their origin. Whole genome sequencing was
114 performed on SCVs from two strains isolated from distinct environments to investigate the
115 potential genetic causes responsible for the SCV phenotypes. We found that mutations affecting

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116 c-di-GMP signalling pathways were responsible for SCV emergence in clinical and
117 environmental strains.
118
119 **RESULTS**

120 **The ability to form SCV-like morphotype colonies is a conserved feature of *Pseudomonas***
121 ***aeruginosa***

122 Culture conditions promoting biofilm formation select for SCVs of *P. aeruginosa* (16,
123 18, 36). To broadly investigate the ability of *P. aeruginosa* to adopt a SCV-like morphotype, we
124 cultured 22 isolates from various origins in static liquid medium for 65 h then spread onto TSA
125 plates to obtain isolated colonies. Six strains were from food samples (meat and fish from
126 markets), six from clinical samples (five from CF patients and the clinical prototypic strain PA14
127 from a burn patient), five from petroleum oil-contaminated soil and five from hospital sinks
128 (drain, splash area and tap) (Table 1, columns 1 and 2). To cover the variety of temperatures
129 relevant to these various habitats, the cultures were incubated in a temperature range varying
130 from 30 to 40°C. At the onset, none of the strains were displaying a SCV phenotype, but after 65
131 h of incubation all isolates diversified in a range of colony morphotypes, including small
132 colonies that appeared typical of SCVs (Fig. 1, for selected strains from each origin). Small
133 colonies emerged in the cultures incubated at all tested temperatures (data not shown).

134 Reported SCVs have an average diameter two to four times smaller than WT colonies.
135 Colonies correspondingly smaller than the parental strains emerged from all 22 strains (Table 1).
136 This result strongly suggests that the ability to produce variant colonies displaying an SCV-like
137 morphotype is a conserved feature of *P. aeruginosa*, regardless of the origin of the strains.
138

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139 **Isolated SCV-like morphotype colonies belong to two distinct clusters**

140 By taking a closer look at the emerged SCV-like morphotypes, we observed that their
141 sizes (Table 1) and overall appearance (Fig. 1) differ. Some colonies were denser, with well-
142 defined round edges and others were more translucent with undefined edges (Fig. 1). We then
143 asked whether these different types of SCV-like morphotypes are indeed *bona fide* SCVs, and if
144 a distinction can be made between them. We focused on five strains from different origins,
145 (Table 1, strains indicated by an asterisk) and isolated the various distinct morphotypic small
146 colonies (SMs for SCV-like morphotype) produced by each following static incubation and
147 plating. Besides their sizes, we looked at several phenotypes typically associated with SCVs:
148 swimming motility defect, biofilm formation and production of EPS, cell aggregation and
149 production of c-di-GMP. Because cell aggregation induces the production of pyoverdine, the
150 fluorescent siderophore of *P. aeruginosa*, while loss of the EPS coding genes, *pel* and *psl,* leads
151 to inhibition of pyoverdine production (41), we used the production of pyoverdine as an indirect
152 measurement of cell aggregation and EPS production. We compiled the phenotypical data for
153 each distinct SMs (Table S1) and performed a principal coordinates analysis (PCoA) based on
154 their colony size, auto-aggregation properties (pyoverdine production), their ability to perform
155 swimming motility, timing of biofilm formation and total biomass of biofilms. In a PCoA, all
156 variables are equally considered to cluster SCVs in significant groups based on their phenotypic
157 profiles and to better understand which SMs are close to each other and could be part of the same
158 clusters. We found that the various distinct SMs generated by the five parental strains clustered
159 in two separate groups (named Cluster 1 and Cluster 2) (Fig. 2). Members of both clusters for the
160 SMs of soil strain 57RP, the sink hospital strain CL-511, the food strain PB PFR11 C2, and the
161 clinical strain FC-AMT0134-9 had phenotypical features that distinguished them from their

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162 parental strain (Fig. 2). Cluster 2 of strain PA14 contained only one isolated SM, but we believe
163 that this is only the result of lower abundance of this form when sampling was performed. These
164 results indicate that two distinct phenotypic types of SCV-like morphotypes emerged under our
165 culture conditions.
166

167 **SMs from Cluster 1 are typical SCVs with a reversible state**

168 SMs belonging to Cluster 1 of each strain share some common features: a reduced
169 swimming motility, and/or a promoted biofilm formation, and/or enhanced auto-aggregation
170 properties (pyoverdine production) as compared with their parental strain (Table S1 and Fig. S1).
171 These features are typical of SCVs described in the literature. Since these phenotypes are
172 regulated by c-di-GMP, we assessed intracellular c-di-GMP levels in selected SMs of Cluster 1.
173 As expected, higher c-di-GMP levels were measured in Cluster 1 SMs than in their parental
174 counterparts, again indicating that Cluster 1 SMs are indeed typical SCVs (Fig. 3). In addition to
175 quantitative PCoA data, we looked at rugosity of SM colonies, a qualitative phenotype
176 traditionally associated with SCVs. While Cluster 1 SMs colonies display a very distinctive
177 rugose surface as compared with their parental counterparts, rugosity appearance was diverse
178 among the strains (Fig. 4). In conclusion, phenotypic characterisation confirms that SMs
179 belonging to Cluster 1 are typical SCVs.

180 Finally, we observed the emergence of spontaneous reversion to a larger, parental-like
181 phenotype, a property typically associated with phase variation. As stated above, on agar plates,
182 reversion to the parental-like morphotype was observed after a 48h incubation at 30°C for SMs
183 belonging to Cluster 1 (Fig. 5). Reversion was revealed as an outgrowth from the original
184 colony, but sometimes only by a change in appearance of the colony surface, as seen for instance

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185 with isolate PB PFR11 C2 (Fig. 5). This reversibility suggested that SCVs could arise from a
186 phase variation process.
187
188 **Cluster 1 SCVs harbor mutation in c-di-GMP pathways**
189 To investigate if SCVs could arise from phase variation, we performed whole genome
190 sequencing of PA14 and 57RP SCVs obtained from independent experiments of 65h static
191 cultivation with the parental strains. The genomes of the parental strains were used as reference
192 for the search for potential mutations in SCVs genomes. Mutations were found in all SCVs
193 (Table 2). Interestingly, they were exclusively detected in genes involved in c-di-GMP
194 metabolism. SCVs randomly selected from the first experiment with the parental strain PA14
195 carry missense single nucleotide polymorphism (SNP) mutations in the *yfiN* gene, while SCVs
196 obtained from the second and third experiment have mutations in the *wsp* cluster, specifically in
197 the *wspA* and *wspF* genes (Table 2). Mutations in the *wsp* cluster are SNPs, resulting in a
198 missense or a stop codon; except for one variant showing a single base deletion leading to a
199 frameshift. SCVs obtained from 57RP carry mutations exclusively in the *wspA* gene;
200 specifically, an in-frame 42-bp deletion (Δ285−298 aa) is present in 12 sequenced SCVs over 13
201 total (Table 2). The other sequenced 57RP SCV also carry a mutation in *wspA* but it is a
202 missense SNP leading to the replacement of a proline residue by a serine residue, potentially also
203 resulting in modulation of WspA activity (Table 2). These mutations in PA14 and 57RP SCVs
204 genomes are likely to be responsible for the increased c-di-GMP levels we measured (Fig. 3).
205 These results indicate that SCV emergence is largely due to mutations resulting in increased c-di-
206 GMP levels. On the other hand, transposon mutants of *wspR* or *yfiN*, resulting in the inactivation
207 of DGCs WspR or YfiN in PA14 do not affect the rate of SCV emergence, suggesting this

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208 phenomenon is regulated by interchangeable DGCs (Fig. S2). Yet, the genetic cause of the SCV
209 phenotype remains elusive: is it always arising from stable mutations or is it a consequence of
210 reversible mutations accounting for the reversion stated above?
211
212
213 **Cluster 1 SCV emergence is due to stable mutations and a second mutation is**
214 **responsible for reversions**
215 Despite the presence of mutations in the SCVs, reversion is still systematically observed
216 on agar plates; this keeps suggesting that their emergence could be regulated by a phase variation
217 mechanism. For each strain tested, reversions of SCVs are obtained directly within the colony by
218 extending the incubation time of the plate (Fig. 5). However, the inoculation of SCVs under non-
219 favorable conditions, *e.g.* cultivation with agitation, did not enable to detect the emergence of
220 revertants, regardless of the strain (data not shown). This result suggests that the mutations that
221 occurred in PA14 and 57RP SCVs were rather stable. To determine if the SCV phenotype was
222 due to a stable or a reversible genetic mutation, whole genome sequencing was performed on
223 reversion outgrowths of SCV PA14 (SM2) and 57RP (SM2) colony (Fig. 5 and Table 2). In the
224 PA14 SCV (SM2) outgrowth, a second SNP mutation was detected downstream of the first
225 mutation in the same gene, *yfiN,* which resulted again in a missense codon. We suppose that this
226 second mutation counterbalances the effect of the first mutation and is responsible for the switch
227 from SCV to another morphotype, probably by inactivating YfiN. In the 57RP SCV (SM2)
228 outgrowth, a second mutation was also detected. However, this mutation is in a different gene,
229 *wspR*, located functionally downstream of the mutated WspA. Thus, regardless of the origin of
230 the strain, reversion was due to a second mutation, indicating that the SCV phenotype is due to

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231 the acquisition of a stable genetic mutation and reversion was not the result of phase variation
232 (Fig. 5 and Table 2).
233

234 **SMs from Cluster 2 display phenotypical heterogeneity**

235 Unlike Cluster 1 SMs, SMs included in Cluster 2 display inter-strain diversity when
236 considering the phenotypes used for the PCoA (Table S1 and Fig. S1). For instance, among
237 Cluster 2 SMs, swimming motility was intermediate between the parental strain and Cluster 1
238 SMs for strains 57RP and PB PFR11 C2 (Table S1 and Fig. S1, A). However, for strains CL-511
239 and FC-AMT0134-9 the swimming motility was increased compared to both Cluster 1 SMs and
240 the parental strains (Table S1 and Fig. S1, A). In addition to PCoA data, c-di-GMP production in
241 Cluster 2 SMs was also variable depending on the parental strain: 57RP Cluster 2 SMs showed
242 higher levels of c-di-GMP compared with both parental strain and Cluster 1 SMs but CL-511
243 Cluster 2 SMs showed higher production of c-di-GMP only compared to the parental strain (Fig.
244 3). Also, Cluster 2 SMs in the food strain PB PFR11 C2 showed similar production of c-di-GMP
245 and Cluster 2 SMs in the clinical strain FC-AMT0134-9 even lower production of c-di-GMP as
246 compared to their parental strain (Fig. 3). Thus, c-di-GMP levels are not a consistent driving
247 feature for SMs belonging to Cluster 2. The appearance of the colony surface of Cluster 2 SMs is
248 also distinct on Congo Red plates, once again depending on the parental strain. Colonies of SM3
249 and SM4 from 57RP display a rugose surface, however less pronounced than for Cluster 1
250 morphotypes (SM1, SM2, SM5 and SM6), in agreement with their reduced autoaggregative
251 properties (Fig. 4 and Fig. S1, D). For the other strains (PA14, PB PFR11 C2, CL-511 and FC-
252 AMT0134-9), SMs from Cluster 2 display a smoother surface on Congo Red agar, closer to the
253 parental strain (Fig. 4). While Cluster 2 SMs show rapid emergence to reproducible phenotypes,

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254 reversion to a larger colonial morphotype akin to WT was only observed for 57RP Cluster 2 SMs
255 and not for the other strains, after 96 h (Fig. 5). All together, these results indicate that, apart
256 from strain 57RP, SMs from Cluster 2 do not exhibit most of the typically described features of
257 SCVs.
258
259 **DISCUSSION**

260 **The ability to switch to the SCV phenotype is a conserved feature among *P.*
261 *aeruginosa* strains, regardless of their origin**

262 SCVs have been mostly reported in the context of human infections, notably from CF
263 individuals. A correlation between the emergence of *P. aeruginosa* SCVs and infection
264 persistence in animal models was established, supporting the idea that the SCV phenotype
265 confers a fitness advantage under chronic infection conditions (42-44). Switch towards the SCV
266 morphotype may represent an adaptation strategy to the hostile environment of the host by
267 increasing resistance to host immunity and antimicrobial treatments (43, 45). However, the
268 emergence of SCVs is not exclusively related to a clinical context. For instance, in 2001 Déziel
269 *et al.* (18) reported the emergence of SCVs in laboratory cultures of a soil *P. aeruginosa* isolate.
270 However, since then, apart from laboratory-grown prototypical strains *P. aeruginosa* PAO1 and
271 PA14, both of clinical origins, no SCVs have been reported from a non-clinical context.
272 Therefore, the question of prevalence remained open: is the ability to adopt a SCV phenotype
273 mostly restricted to clinical isolates or clinical context, from chronic infections - or not?

274 Here, we investigated the distribution of a SCV-based adaptative strategy in *P.*
275 *aeruginosa* by screening 22 strains from diverse origins. Selective conditions were achieved by
276 static cultivation, a culture condition that generates different microenvironments, as seen by the

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277 formation of a pellicle biofilm at the air-liquid interface. Plating of bacteria from static cultures
278 of all 22 strains resulted in the formation of small colonies with sizes similar to SCVs described
279 in other studies (16, 18). However, SCVs are not exclusively defined by the smaller size of their
280 colonies. SCVs are also often identified based on the rugosity of the colony formed on Congo
281 Red agar plates, hence the alternate name RSCVs for Rugose Small Colony Variants (14, 24,
282 43). Nevertheless, rugosity is a subjective feature, and its description may vary according to the
283 observer and culture conditions. Indeed, we have observed that the rugosity level varies between
284 strains. This might be especially true for strains originating from diverse environments, as in the
285 present study. Thus, we decided to take advantage of the various additional phenotypes described
286 for SCVs to ascertain their identity (Fig. 6). To this end, we focused on five strains representing
287 diverse environmental origins. Based on their phenotypic features, the small colonies obtained
288 from each parental strain were clustered into two distinct groups. Small colonies classified in
289 Cluster 1 shared several inter-strain phenotypic features, including reversion visible after 48h.
290 Based on known features, these small colonies can be defined as typical SCVs, validating that
291 SCVs emerge from *P. aeruginosa* isolated from any origins. Thus, the ability to switch to the
292 SCV phenotype appears an intrinsic feature of the species.

293 SCVs have always been isolated from biofilm-promoting conditions or from
294 environments where biofilms thrive (16, 39, 40). SCVs are especially prone to adherence and
295 biofilm formation (18, 36, 39). The attached mode of growth (biofilm) is a widespread lifestyle
296 in all types of environments (46-48). Biofilms are protective barriers for their bacterial
297 components and increase tolerance to antimicrobials as compared to free-living bacterial cells,
298 and enhance the ability to survive in extreme conditions, such as desiccation (49-51). Thus, one
299 can easily conceive that the switch to the SCV phenotype confers a significant advantage for

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300 colonization of various ecological niches, accounting for the apparently conserved rapid
301 switching to the SCV phenotype.
302
303 **The SCV phenotype results from a rapidly acquired stable genetic mutation in c-di-**
304 **GMP systems**
305 Intracellular c-di-GMP levels regulate all phenotypes associated with SCVs: EPS
306 production, motility, adherence, etc. (33-35). It is obvious that c-di-GMP thus plays a major role
307 in the regulation of the SCV phenotype, but the role of c-di-GMP remains elusive in the
308 mechanism of emergence. Several studies have reported that SCVs display mutations in genes
309 involved in regulation of c-di-GMP level, particularly genes included in the YfiBNR and Wsp
310 pathways (14, 24, 43). These mutations lead to the activation of the DGC and subsequent
311 increase in c-di-GMP levels, either due to inactivation of the DGC repressor or constitutive
312 activity of the chemosensory protein at the top of the signal transduction pathway. However,
313 none of these studies were based on environmental strains, which is relevant since *P. aeruginosa*
314 is naturally a saprophyte. Thus, involvement of c-di-GMP in the regulation and/or emergence of
315 the SCV phenotype from an environmental parental strain remains elusive. By sequencing the
316 whole genome of cluster 1 SCVs obtained from the prototypical clinical strain *P. aeruginosa*
317 PA14 and the environmental strain *P. aeruginosa* 57RP, we wanted to determine if
318 environmental strains can also switch to the SCV phenotype by using the same mechanism as
319 PA14. We detected stable acquired mutations in all the PA14 SCVs genomes we sequenced (14,
320 24). However, the previous reports have concluded on the stability of the mutation in PA14 SCV
321 by using genetic complementation and observing reversion to parental-like morphotype (14, 24).
322 Here, we present the first study that conclude on the stability of mutations based on the whole

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323 genome sequencing of revertants, highlighting a second spontaneous mutation responsible for the
324 switch of morphotype observed.

325 In a previous report, studying genetic evolution of PA14 upon infection in an animal
326 model, Gloag *et al*. found that all PA14 SCVs displayed driver mutations in the Wsp pathway,
327 mainly in *wspA* and few in *wspF*. Wsp is a chemosensory pathway, activated by surface sensing
328 from WspA, and ultimately leading to the activation of the diguanylate cyclase WspR which
329 catalyses the synthesis of c-di-GMP. This activation loop is regulated by the repressor WspF that
330 acts to reset the system upon phosphorylation by WspE (28, 52). Here, PA14 SCVs from our
331 standing culture conditions acquired mutations in genes of the Wsp pathway, *wspA* and *wspF*,
332 but also in *yfiN*. The distinct membrane-integral DGC YfiN belongs to the YfiBNR pathway.
333 YfiN activity is inversely controlled by the small periplasmic protein YfiR (repression) and the
334 outer membrane protein YfiB (43, 53). While a previous study has reported the importance of the
335 *yfiBNR* operon in emergence of SCVs in *P. aeruginosa* PAO1, our results confirm the
336 importance of *yfiBNR* for SCV emergence in PA14 as well (43). The interest is emphasized by
337 the phylogenetic distance between PAO1 and PA14 strains, belonging to two distinct
338 phylogenetic groups of *P. aeruginosa* (54). Importantly, unlike in this previous study, the *yfiBNR*
339 mutation in PA14 genome leading to SCV phenotype arose spontaneously. In PAO1, targeted
340 mutation of the gene encoding the DGC inhibitor YfiR led to SCV phenotype (43). Here we
341 present the first report of a spontaneous mutation directly in the gene coding the DGC itself,
342 *yfiN*, leading to the SCV phenotype. Surprisingly, this mutation results in an increased c-di-GMP
343 level, maybe due to a distinct steric hindrance following the replacement of a cysteine residue by
344 a serine.

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345 Apart from *P. aeruginosa* PAO1 and PA14, sometimes considered as laboratory strains
346 rather than clinical strains, this is the first report studying the mechanism of SCV phenotype
347 emergence in *P. aeruginosa* environmental strains, such as 57RP. By sequencing the genomes of
348 several independently evolved 57RP SCVs, we found that the same mutation frequently occurred
349 in *wspA*. Interestingly, the exact same *wspA* Δ285-298 deletion was also the most common
350 mutation detected in PA14 SCVs upon infection of a murine chronic model (24). Several studies
351 have reported mutations in this particular region (14, 55, 56), probably because this region may
352 be hypermutable (24). Yet, it is striking that the deletion was the same in SCVs from PA14 and
353 57RP (24), especially since SCV selection conditions were completely very different. Since this
354 deleted sequence is flanked by repeated inverted sequences, it could be a mobile element (24).
355 However, we were not able to find this sequence at any other location in the parental strain
356 genomes, nor in revertants genomes, suggesting that this may not be a reversible deletion. More
357 so, the deletion was stable since reversion was due to a second mutation in a downstream gene of
358 the *wsp* operon. This deletion was proposed to lead to a constitutive signaling and autoinduction
359 of the Wsp pathway by alteration of methylation/demethylation of WspA, which would result in
360 an increase in c-di-GMP production (24). While this is the first report of a Wsp mutation leading
361 to SCV emergence in an environmental *P. aeruginosa* strain, it should be emphasized that a
362 mutation in the Wsp pathway leading to the SCV phenotype was first detected in the
363 environmental strain *P. fluorescens* Pf0-1 (55, 57). All together, these results indicate that c-di-
364 GMP plays a central role in SCVs emergence, in strains of both clinical and environmental
365 origins.

366 Interestingly, only one mutation was identified in the genomes of *P. aeruginosa* PA14
367 and 57RP SCVs. The sole other study which has also detected mutations having appeared

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368 spontaneously in *P. aeruginosa* PA14 SCV reported secondary mutations in the SCVs genome
369 (24). Also, after a unique 65h incubation of the parental strain under static culture conditions,
370 SCVs from both *P. aeruginosa* PA14 and 57RP represented 44.4 % and 18.7 % of the total
371 population, respectively. This indicate that, regardless of the strain origin, mutants in c-di-GMP
372 pathways are selected *in vitro* to adapt to specific conditions and switch to the SCV phenotype
373 (Fig. 6). Among all the c-di-GMP pathways known in *P. aeruginosa*, Wsp and YfiBNR seem to
374 be preferred pathways involved in SCV emergence. However, although the c-di-GMP increase
375 resulting from alteration in Wsp and YfiBNR pathways are responsible for SCVs emergence, the
376 opposite was not true. Inactivation of the DGCs WspR and YfiN, resulting in inability to produce
377 c-di-GMP through this pathway did not affect the rate of emergence of SCVs. Thus, regulation
378 of SCV emergence through c-di-GMP mechanisms could be based on interchangeable DGCs,
379 ready to take over the inactivity of one of the pathways.
380

381 **SCVs could also emerge from a phase variation mechanism, undetectable under**
382 **laboratory conditions**

383 Phase variation is a common phenomenon among Gram-negative bacteria and is typical
384 of bacteria thriving in heterogeneous ecological niches (21, 22, 58), notably *P. aeruginosa* (19).
385 Unlike acquisition of stable mutations, phase variation mechanism represents a significant
386 advantage for the rapid adaptation to sudden changes in the environment (59, 60). Indeed, phase
387 variation mechanisms lead to emergence of a heterogeneous population in which the best
388 suitable phenotype will multiply until the conditions fluctuate again and the selected phenotypes
389 revert to another phenotype.

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390 Although SCVs are due to stable genetic mutations under our experimental conditions,
391 i.e. irreversible mutations, we cannot exclude that the adoption of the SCV phenotype could also
392 rise from a reversible phase variation regulated mechanism in natural habitats. Several reports
393 support this hypothesis. First, phenotypes traditionally related to SCV (motility, aggregation) are
394 often regulated by phase variation mechanisms (21). In addition, reversible adaptation
395 mechanisms are based on transitory DNA rearrangements (gene conversion, genomic inversion,
396 DNA recombination..) and lead to variation in gene expression (20). Indeed, one recent study
397 reports a large genomic inversion in *P. aeruginosa* SCVs (61). Finally, reversion of SCVs has
398 been observed several times and could be due to phase variation instead of emergence of a
399 second mutation, but no whole genome sequencing has been performed to conclude (15, 16, 18).
400 Yet, SCVs reversion occurred toward a phenotype likely different from the parental morphotype
401 (16, 23), suggesting that regulation is not necessarily an ON/OFF switch on a particular locus
402 and could be due to a secondary mutation in the genome.
403 Under our conditions, SCVs from phase variation mechanism could have arisen and were
404 undetectable in our conditions with our technique. Two elements could explain this limitation:
405 (1) the “reversible” SCVs are present but in undetectable quantities to be observed after sampling
406 and agar spreading or (2) “reversible” SCVs were present but reverted to another morphotype
407 when the samples were spread on agar plates. Indeed, various phenotypes were observed on agar
408 plates after 65h of standing incubation, likely to have emerged in the static liquid culture but they
409 could have also emerged directly on the agar plate. To verify this hypothesis and verify that
410 SCVs can also arise from a reversible mechanism, it would be interesting to follow the
411 emergence of SCVs in the static culture using a detectable marker.
412

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413 **Small colonies are not necessarily SCVs, nor variants**

414 During our experiments with static cultures, we observed several small-colony
415 morphotypes. Based on our PCoA analysis, a proportion of them were clustered in two distinct
416 groups (Fig. 2). Except for strain 57RP, the SMs from Cluster 2 did not display clear reversion
417 after 48 h on solid medium (data not shown). However, SMs from Cluster 2 could still be able to
418 revert in conditions outside the ones tested in our study. Also, their rate of emergence seemed too
419 high for mutants (Table 1). Thus, we wonder if cluster 2 SMs should be identified as variants
420 based on our criteria.

421 In contrast with SMs from Cluster 1, SMs from Cluster 2 showed inter-strain
422 heterogeneous features. We observed a large diversity of morphotypes on plates prepared from
423 our static cultures. Among them, large colonies also displayed features similar to revertants (16).
424 This observation indicates that reversion could have occurred in the static liquid cultures, and
425 intermediate forms could consequently be isolated. Maybe several mechanisms are acting in
426 parallel to induce the phenotypical diversity we observed, thus promoting the selection of the
427 best adapted subpopulation.

428 The SCV phenotype has been linked to the persistence of *P. aeruginosa* in the context of
429 infections in a human host, notably because of its increased resistance against antimicrobials and
430 host immunity. However, we have demonstrated here that strains isolated from soil, food and
431 hospital environments can also readily adopt a SCV phenotype. This indicates that the ability of
432 *P. aeruginosa* to form SCVs is a conserved feature of this species, and SCVs emergence is not
433 exclusively related to the pressure of the infection-related clinical environment. This is the first
434 report of high prevalence of SCVs among *P. aeruginosa* strains, regardless of the origin of the
435 isolates. The SCVs identified showed specific mutations in genes related to regulation of the c-

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436 di-GMP intracellular levels. The Wsp and YfiBNR systems were the primary pathways used to
437 increase c-di-GMP level and switch to SCV phenotype. Emergence of SCV in the various
438 habitats allows *P. aeruginosa* to rapidly adapt and persist under diverse environmental
439 conditions, accounting for its versatility and persistence. A deeper comprehension of the
440 adaptation strategy used by *P. aeruginosa* could ultimately provide innovative strategies for
441 eradication of this opportunistic pathogen of public concern.

442

443 MATERIALS AND METHODS

444 Bacterial strains and growth conditions

445 Bacterial strains are listed in Table 1 and their specific origin are listed in Table S2. In this study,
446 the term "parental strain" designs the original strain used to evolve other morphotypes in static
447 cultures, including SCVs. Strains were grown in tryptic soy broth (TSB; BD), at 37°C in a TC-7
448 roller drum (New Brunswick Scientific) at 240 rpm for the parental strains and at 30°C in an
449 Infors incubator (Multitron Pro) at 180 rpm (angled tubes) for the isolated evolved morphotypes.
450 Static cultures were inoculated with the parental strain at an initial OD_{600} of 0.05 and incubated
451 at 30, 30.9, 32.2, 33.9, 36.3, 38, or 40°C for 65 hours. Cultures were then spread on tryptic soy
452 2% agar plates (TS-Agar; AlphaBiosciences), unless stated otherwise. Two percent agar was
453 utilized to limit expansion of colonies and improve isolation of the distinct morphotypes.

454

455 Bradford protein assay

456 Due to the highly aggregative properties of SCVs, OD_{600} measurements were not appropriate to
457 evaluate growth of some of the isolated evolved morphotypes. Instead, the Bradford protein
458 assay was used to quantify the concentration of total proteins in all our samples. Pellets from 1

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459 ml of culture were resuspended in 1 ml 0.1 N NaOH and incubated 1 h at 70°C. Protein
460 concentrations were measured on samples according to the manufacturer guidelines for the
461 Bradford reagent (Alfa Aesar).
462
463 **Phenotypic tests**
464 Overnight (O/N) cultures of parental strains and their isolated morphotypes were grown at 30°C
465 in an Infors incubator (Multitron Pro) at 180 rpm in angled tubes. Since biofilm formation
466 occurred in cultures, they were transferred to clean tubes to perform experiments or Bradford
467 protein quantifications. Statistical analyses were achieved using Ordinary one-way analysis of
468 variance (ANOVA). Each phenotypic test was performed in technical triplicates.
469
470 **Morphology on Congo red plates**
471 A 1% Congo red solution in water (Fisher SCIENTIFIC) was added to TS-Agar 2% to a final
472 concentration of 0.1%. Ten µL of culture were spotted on the plates. Plates were incubated at
473 30°C and observed after 24 h, 48 h and 96 h. Plates were observed with a binocular StemiDV4
474 (Zeiss) and photos were taken with the camera DMC-ZS60 (Panasomic Lumix).
475
476 **Swimming motility tests**
477 Swim plates (20 mM NH_4Cl, 12 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 0.5% Casamino
478 acids (CAA), 0.3% Bacto-Agar (BD), supplemented with 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 11
479 mM dextrose) were prepared and dried for 15 min under the flow of a Biosafety Cabinet. A
480 volume of 2.5 µL of culture was inoculated in the agar. Plates were incubated for 20 hours at

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481 30°C. Swimming ability was assessed by measuring the area (mm^2) of the turbid circular zone
482 using ImageJ. All experiments were performed in triplicates.
483
484 **Biofilm formation**
485 Microtiter (96-well) plates containing 1/10 TSB supplemented with 0.5% CAA were inoculated
486 from a transferred O/N culture in order to obtain a starting concentration of 70 mM proteins.
487 Each sample was inoculated in five different wells. Plates were incubated at 30°C without
488 agitation. After 6 and 24 h, plates were rinsed thoroughly with distilled water and 200 µL of a
489 1% crystal violet solution was added to each well. After 15 minutes of incubation at room
490 temperature, plates were rinsed thoroughly with distilled water and the dye was solubilized in
491 300 µL in 30% acetic acid. The absorbance was measured at 595 nm with a microplate reader
492 (Cytation3, Biotek). Initiation of biofilm formation was calculated as the % of biofilm formed
493 after 6 h of incubation compared with total biofilm formed after 24 h incubation. Total biomass
494 of the biofilm was calculated as the amount of biofilm formed after 24 h, measured by crystal
495 violet absorbance at 595 nm after 24 h of incubation.
496
497 **Pyoverdine production**
498 Overproduction of pyoverdine was previously noted as a feature of strain 57RP SCVs (18). We
499 confirmed that a SCV from PA14 expresses high fluorescence level at the wavelength of
500 pyoverdine emission, likely to account for cell aggregation and EPS overproduction. An SCV
501 isolated from a PA14 *pvdD* mutant (62), which is no longer able to produce pyoverdine, showed
502 lower fluorescence levels, similar to parental colonies, confirming that [1] pyoverdine production
503 is responsible for the fluorescence detected and [2] measured fluorescence is correlated with

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504 SCV aggregation properties (Fig. S3). To measure pyoverdine production, black 96-well plates
505 (Greiner) were filled with 200 µL of culture. Fluorescence was measured at wavelengths 390
506 nm/530 nm excitation/emission using a multimode microplate reader (Cytation3, Biotek).
507

508 **C-di-GMP quantification**

509 Intracellular levels of c-di-GMP were assessed with the fluorescence-based biosensor pCdrA-
510 gfpC (63, 64), acquired as addgene plasmid #111614; http://n2t.net/addgene:111614 ;
511 RRID:Addgene_111614. Purified plasmids were transformed by electroporation in evolved
512 morphotypes obtained from static cultures (65). Transformants were selected on TS-Agar 2%
513 supplemented with 100 µg/ml gentamycin. Three clones for each transformed morphotypes were
514 cultured in TSB supplemented with gentamycin 100 µg/ml. Cultures were washed twice in fresh
515 TSB to get rid of a potential non-specific fluorescence due to secreted fluorescent pigments as
516 pyoverdine. Fluorescence was measured using a Cytation3 microplate reader (BioTek) at 490
517 nm/515 nm (excitation/emission) in black 96-well plates (Greiner). The non-transformed strain
518 was used as a control. Fluorescence from the control was subtracted from the fluorescence signal
519 for the transformed strains.
520

521 **PCoA analysis**

522 Colonies identified as SMs compared with their parental isolate (see Results) were used to
523 perform a principal coordinate analysis (PCoA). Statistical analyses were performed using
524 RStudio software version 1.3.1093 (66) with normalised data showed in Table S1. A Euclidean
525 distance matrix was used to generate a clustering of the bacterial isolates according to their
526 phenotypical profile. A Similarity Profile Analysis (simprof) was performed to determine the

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527 number of significant clusters produced using hclust with the assumption of no *a priori* groups.
528 Significant clusters were considerate when at least two evolved morphotypes constituted it.
529

530 **Sequencing and analysis**

531 To analyse SCVs genomes and compare them with corresponding parental strain genomes,
532 genomic DNA was extracted using the EasyPure® Genomic DNA Kit (Transgen Biotech),
533 according to the manufacturer's protocol from 200 µL of O/N culture of colony variants in TSB.
534 Clonal DNA was sequenced on the Illumina NextSeq 2000 at the Microbial Genome Sequencing
535 center (MiGS); 2x151 bp sequencing paired end reads were trimmed and quality filtered using
536 fastp v0.23.2 (67). Filtered reads were assembled using Skesa through Shovill v1.1.0 and
537 annotated using prokka v1.14.6 to be used as reference sequences (68). The reads were then used
538 for variant calling using snippy v4.6.0 (https://github.com/tseemann/snippy) using default
539 settings.
540 Presence of a 42 bp deletion in *wspA* for additional 57RP colony variants DNA was confirmed
541 by amplifying a 200 bp PCR fragment with primers F-CGGAGACTTCGCTCATGGT and R-
542 AGAGCTCAAGGGCCTGGT. The detection of amplified products was performed on a 2%
543 agarose gel electrophoresis.
544

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553

554 AB and ED conceived the project, contributed to experimental design and interpreted
555 results. AB, MCG and MT contributed to data acquisition. AV and AB analysed sequencing. AB
556 wrote the manuscript. MCG and ED reviewed and edited the manuscript.
557

558

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775 **FIGURE LEGENDS**

776

777 **Fig. 1. Small colonies of *Pseudomonas aeruginosa* emerge in static cultures from strains**
778 **isolated from various origins.**
779 Parental strains were inoculated under static liquid conditions in TSB for 65 hours and spread
780 onto TS-Agar 2% plates. Black arrows indicate smaller colonies. White arrows indicate parent-
781 like colony. Scale bars represent 5 mm.

782

783 **Fig. 2. Small colonies isolated from static cultures are clustered in two separate groups**
784 **according to their phenotypic features.**
785 PCoA analysis were performed with a matrix composed of data obtained from the phenotypic
786 analyses (swimming, biofilm formation, and pyoverdine production) for the parental strain and
787 distinct small colonies isolated from static cultures with a diameter at least two times smaller
788 than parental strain (Table S1). All variables were equally considered to cluster colonies in
789 significant groups based on their phenotypic profiles. Each point represents a small colony
790 isolated from the static cultures and has a name code composed of SMx standing for **S**mall
791 **M**orphotype where x is an arbitrary number attributed during the isolation of the colonies. The
792 identification of statistically distinctive clusters was performed using simprof tests and hclust.

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794 **Fig. 3. c-di-GMP production is altered for SMs from Cluster 1 and 2 compared with their**
795 **respective parental strain.** c-di-GMP production was measured with the fluorescent-based
796 biosensor pCdrA-gfp on O/N washed cultures. The values are means ± standard deviations (error
797 bars) for selected transformed morphotypes belonging to each cluster: transformed morphotypes
798 were SM2 and SM6 (cluster 1) and SM4 (cluster 2) for strain 57RP; SM4 and SM5 (cluster 1)
799 for strain PA14; SM8 and SM9 (cluster 1) and SM10 (cluster 2) for strain CL-511; SM1 and
800 SM2 (cluster 1) and SM3 and SM6 (cluster 2) for strain PB PFRC11 2; SM9 (cluster 1) and SM5
801 and SM7 (cluster 2) for strain FC-AMT0134-9. Three transformants of each SMs were
802 considered in the calculation of mean and standard deviations. Stars represents the statistical
803 significance of the results calculated by an Ordinary one-way analysis of variance (ANOVA),
804 ****, P Value ≤ 0.0001; **, P Value ≤ 0.01; ns, not significant. Data are normalized between
805 them based on their parental strain.

806

807 **Fig. 4. Appearance of colonies for the parental isolates and SMs from Cluster 1 and Cluster**
808 **2 on Congo Red plates.** The SM shown for each cluster is representative of all the SMs included
809 in one cluster since they have a similar appearance. Plates were observed with a binocular
810 StemiDV4 (Zeiss) and photos were taken with a DMC-ZS60 camera (Panasonic Lumix), after 24
811 h of incubation at 30°C.

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813 **Fig. 5. Reversion occurs on solid media for specific morphotypes after 48 h incubation.** Ten
814 µl of a culture of parental strain or a cluster representative morphotype (SMs) were dropped on
815 0.1% congo red TS-Agar 2% plates. Plates were observed with a binocular StemiDV4 (Zeiss)
816 and photos were taken with the camera DMC-ZS60 (Panasonic Lumix), after 24 h, 48 h and 96 h
817 of incubation at 30°C. Scale bars represent 5 mm.
818
819 **Fig. 6. Schematic summary of SCV features compare to parental strain**
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821 **Table 1. Colony diameters and prevalence of parental isolates and their static liquid culture**
822 **evolved small morphotypes.**
823

Strain	***P. aeruginosa***	Morphotype[a]	Colony diam. (mm)[b]	Ref	SMs %
Clinical strains	FC-AMT 0102-8	**parental isolate**	**1.57**	(69)	
		SCV-like morphotypes	0.65 ±0.09		8.14 ±4.47
	FC-AMT 0127-13	**parental isolate**	**2.24**	(69)	
		SCV-like morphotypes	0.63 ±0.15		99.2 ±0.41
	FC-AMT 0134-9 *	**parental isolate**	**4.21**	(69)	
		SCV-like morphotypes	0.83 ±0.26		6.91 ±5.95
	FC-AMT 0127-2	**parental isolate**	**2.19**	(69)	
		SCV-like morphotypes	0.73 ±0.18		19.3 ±8.66
	FC-AMT 0166-22	**parental isolate**	**2.27**	(69)	
		SCV-like morphotypes	0.74 ±0.22		6.41 ±3.36
	ED14/PA14*	**parental isolate**	**3.16**	(70)	
		SCV-like morphotypes	1.24 ±0.14		44.4 ±9.62
Food strains	ABO VB50 C1	**parental isolate**	**4.50**	(71)	
		SCV-like morphotypes	0.63 ±0.39		6.44 ±4.06
	BG VB5 C2	**parental isolate**	**4.53**	(71)	
		SCV-like morphotypes	1.12 ±0.17		18.7 ±5.63
	PB PFR11 C2*	**parental isolate**	**2.96**	(71)	
		SCV-like morphotypes	1.17 ±0.24		17.4 ±7.98
	ABO PF5 C1	**parental isolate**	**2.38**	(71)	
		SCV-like morphotypes	0.84 ±0.23		5.63 ±0.20
	BG VB11 C1	**parental isolate**	**2.28**	(71)	
		SCV-like morphotypes	0.93 ±0.17		4.87 ±1.87
	ADJ VB12 C1	**parental isolate**	**2.30**	(71)	
		SCV-like morphotypes	0.91 ±0.27		7.38 ±3.22
Soil strains	19SJV	**parental isolate**	**3.55**	(72)	
		SCV-like morphotypes	1.01 ±0.32		25.9 ±10.6
	34JR	**parental isolate**	**7.20**	(72)	
		SCV-like morphotypes	1.78 ±1.08		4.51 ±1.33
	57RP *	**parental isolate**	**2.61**	(72)	
		SCV-like morphotypes	1.07 ±0.24		18.7 ±8.79
	18G	**parental isolate**	**10.14**	(72)	
		SCV-like morphotypes	1.76 ±1.45		11.1 ±7.69
	PG201	**parental isolate**	**6.08**	(73)	
		SCV-like morphotypes	1.64 ±0.86		20.4 ±14.9
Hospital sink strains	CL-511 *	**parental isolate**	**7.97**	(74)	
		SCV-like morphotypes	1.56 ±0.45		50.5 ±29.2
	CL-542a	**parental isolate**	**2.47**	(74)	
		SCV-like morphotypes	0.95 ±0.22		20.1 ±5.29
	CL-534a	**parental isolate**	**2.52**	(74)	
		SCV-like morphotypes	0.72 ±0.33		7.58 ±2.29
	CL-547b	**parental isolate**	**3.32**	(74)	
		SCV-like morphotypes	0.97 ±0.41		6.72 ±0.73
	PAO303	**parental isolate**	**3.63**	(75)	
		SCV-like morphotypes	1.00 ±0.55		5.29 ±2.09

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824 [a] colonies were considered to be SCV-like morphotype when their diameter was at least half that
825 of the parental isolate
826 [b] average diameters of the small colonies
827 [*] strains marked with an asterisk were selected for further phenotypic characterization
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844 **Table 2. Mutations identified in 57RP and PA14 SCVs and their revertant**

SCV sequencing (reference genome = parental)					Revertant sequencing (reference genome = parental)		
Strain	Exp. no.[a]	SCV no.	Gene	Mutation[b]	Name	Gene	Mutation
57RP	1	1	*wspA*	del 285-298			
		2	*wspA*	del 285-298	**Revertant- SCV no. 2**	*wspA*	del 285-298
		3	*wspA*	del 285-298		*wspR*	Leu71ms
		4	*wspA*	del 285-298			
		5	*wspA*	Pro479ms			
	2	6	*wspA*	del 285-298[c]			
		7		del 285-298[c]			
		8		del 285-298[c]			
	3	9	*wspA*	del 285-298[c]			
		11		del 285-298[c]			
		12		del 285-298[c]			
	4	13	*wspA*	del 285-298[c]			
		14		del 285-298[c]			
		15		del 285-298[c]			
PA14	1	1	*yfiN*	Cys166ms			
		2	*yfiN*	Cys166ms	**Revertant- SCV no. 2**	*yfiN*	Cys166ms
		3	*yfiN*	Cys166ms		*yfiN*	Asp304ms
	2	4	*wspF*	Val318fs			
	3	5	*wspF*	Gln297ns			
	4	6	*wspA*	Ala422ms			

845 [a] Experiment number – each experiments are performed individually from a distinct inoculum of

846 the parental strain

847 [b] deleted amino acid residues are indicated. del, deletion; ms, missense; fs, frameshift; ns,

848 nonsense

849 [c] deletion was detected by PCR and whole genome was not sequenced

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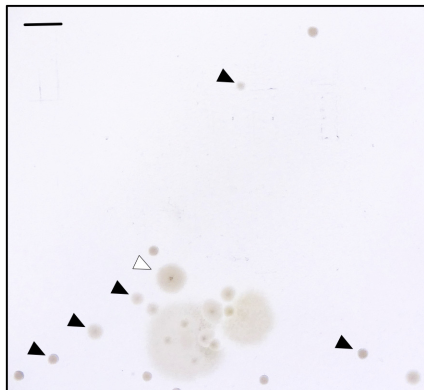
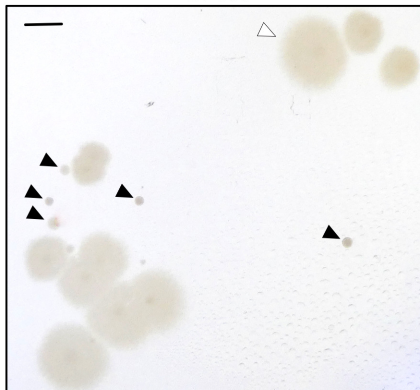
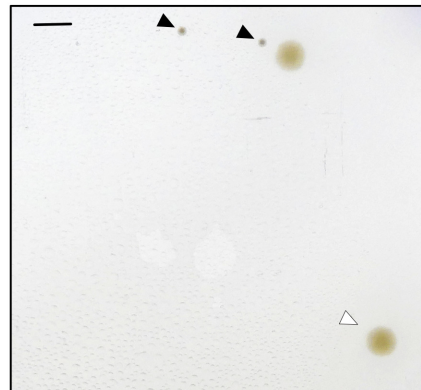
Soil strain
57RP



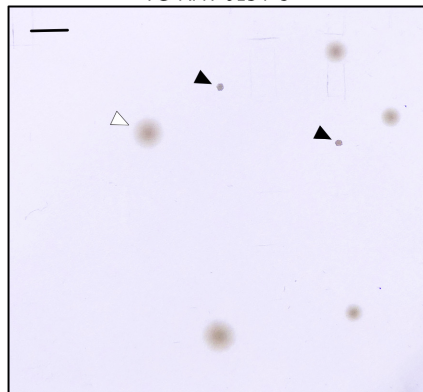
Hospital sink strain
CL-511



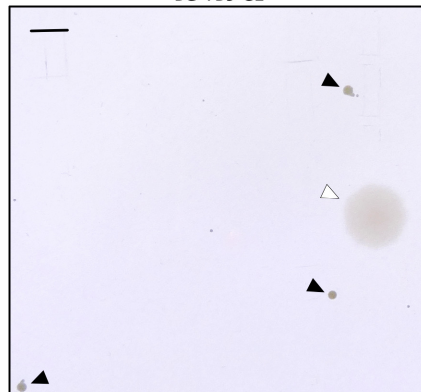
Prototypic clinical strain
PA14



Clinical strain
FC-AMT 0134-9



Food strain
BG VB5 C2



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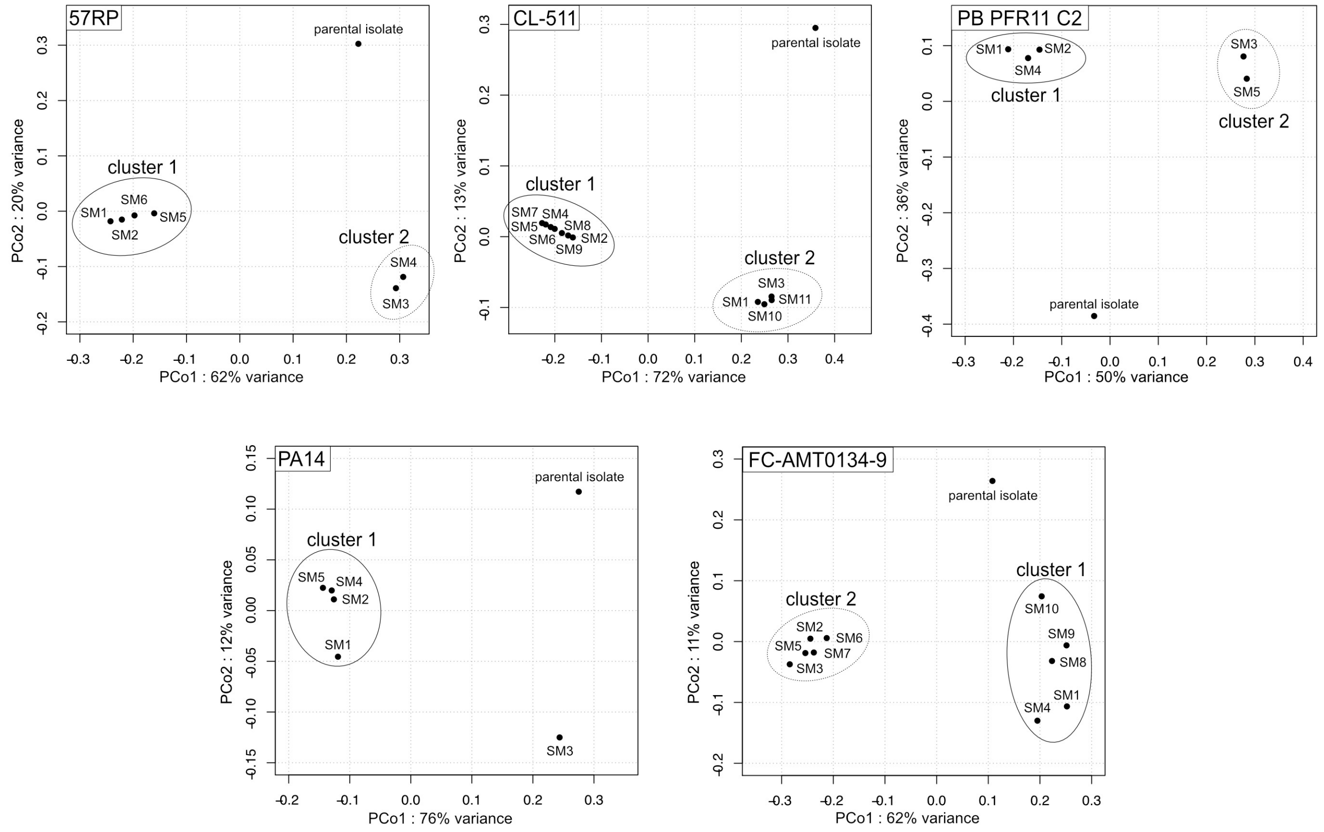

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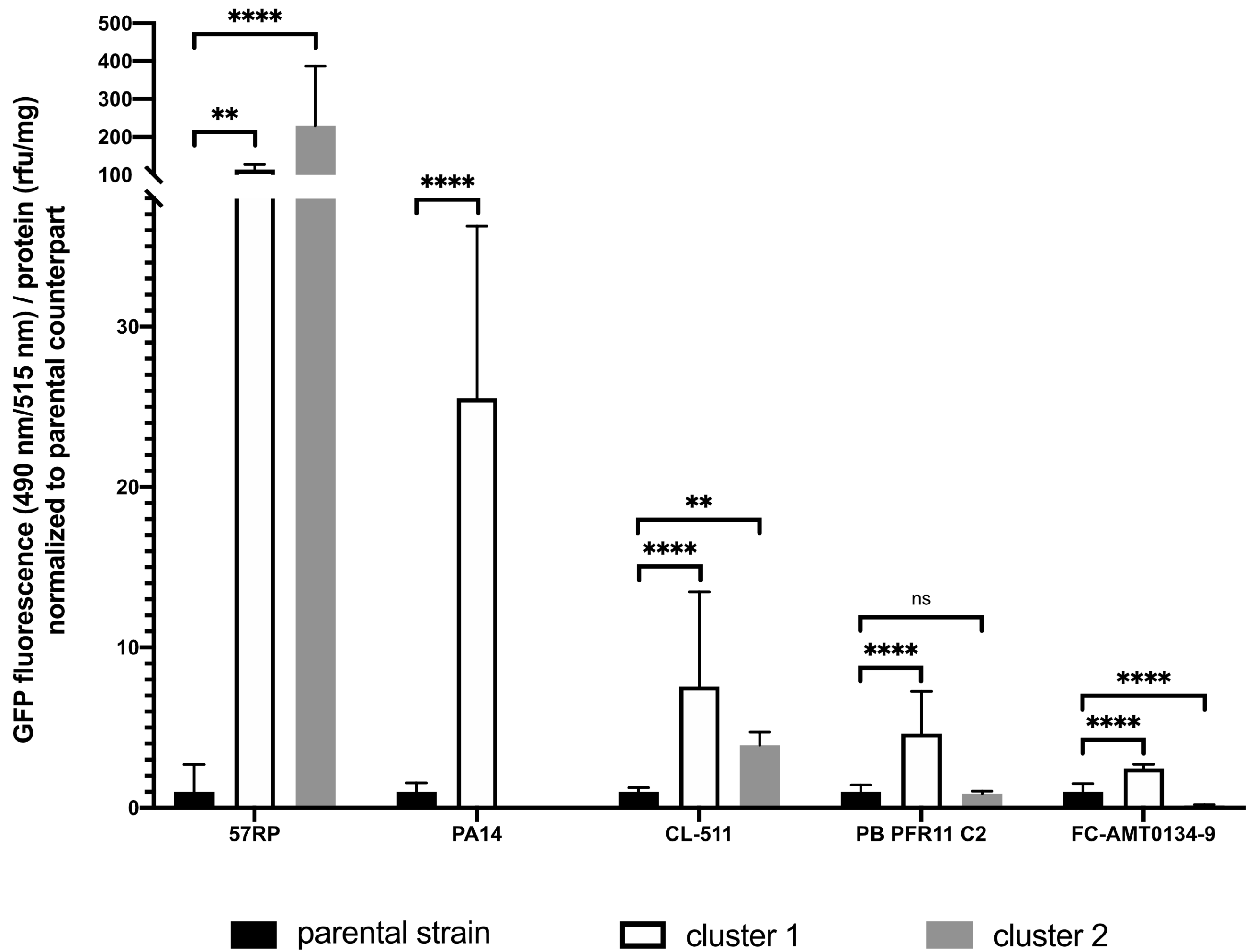

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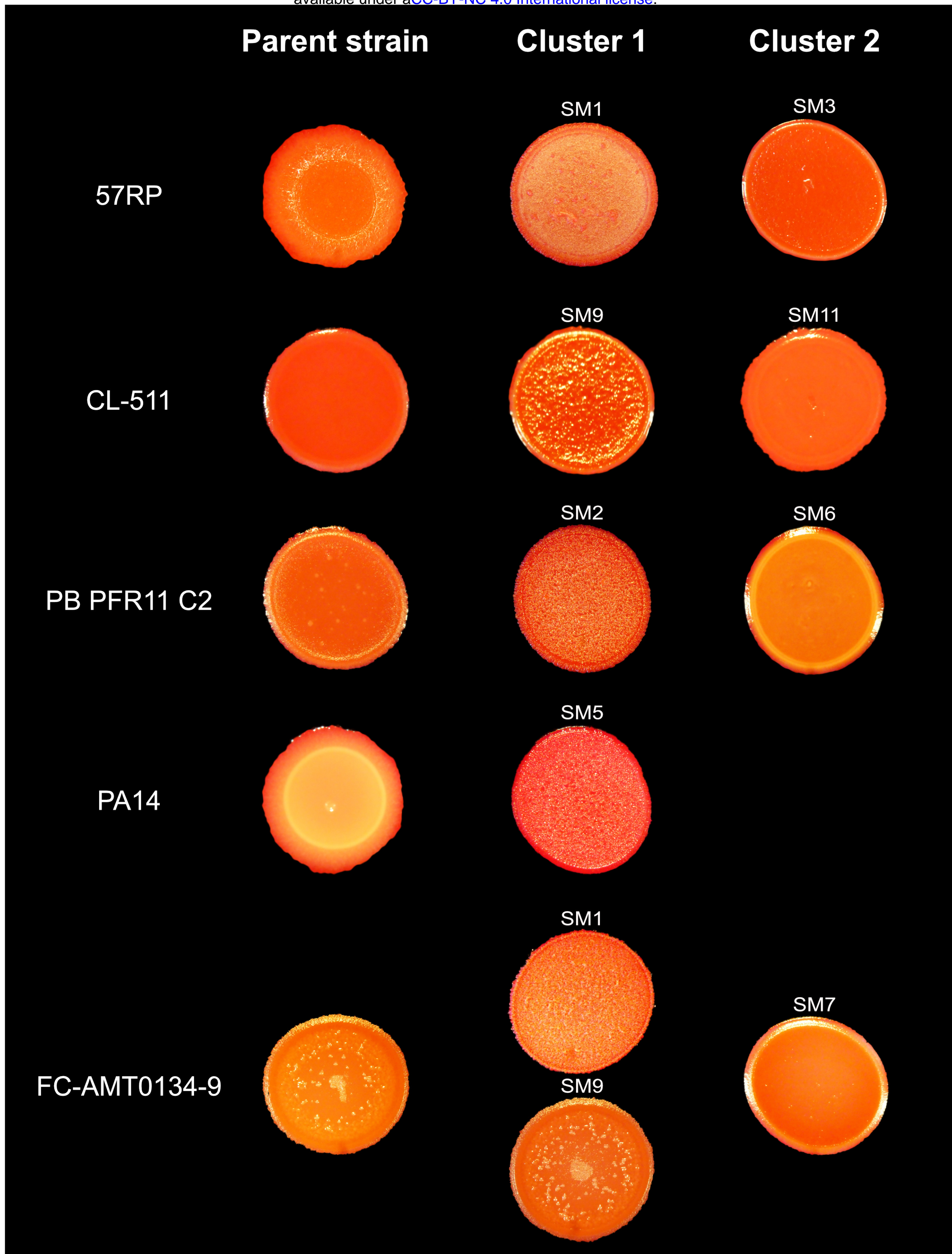

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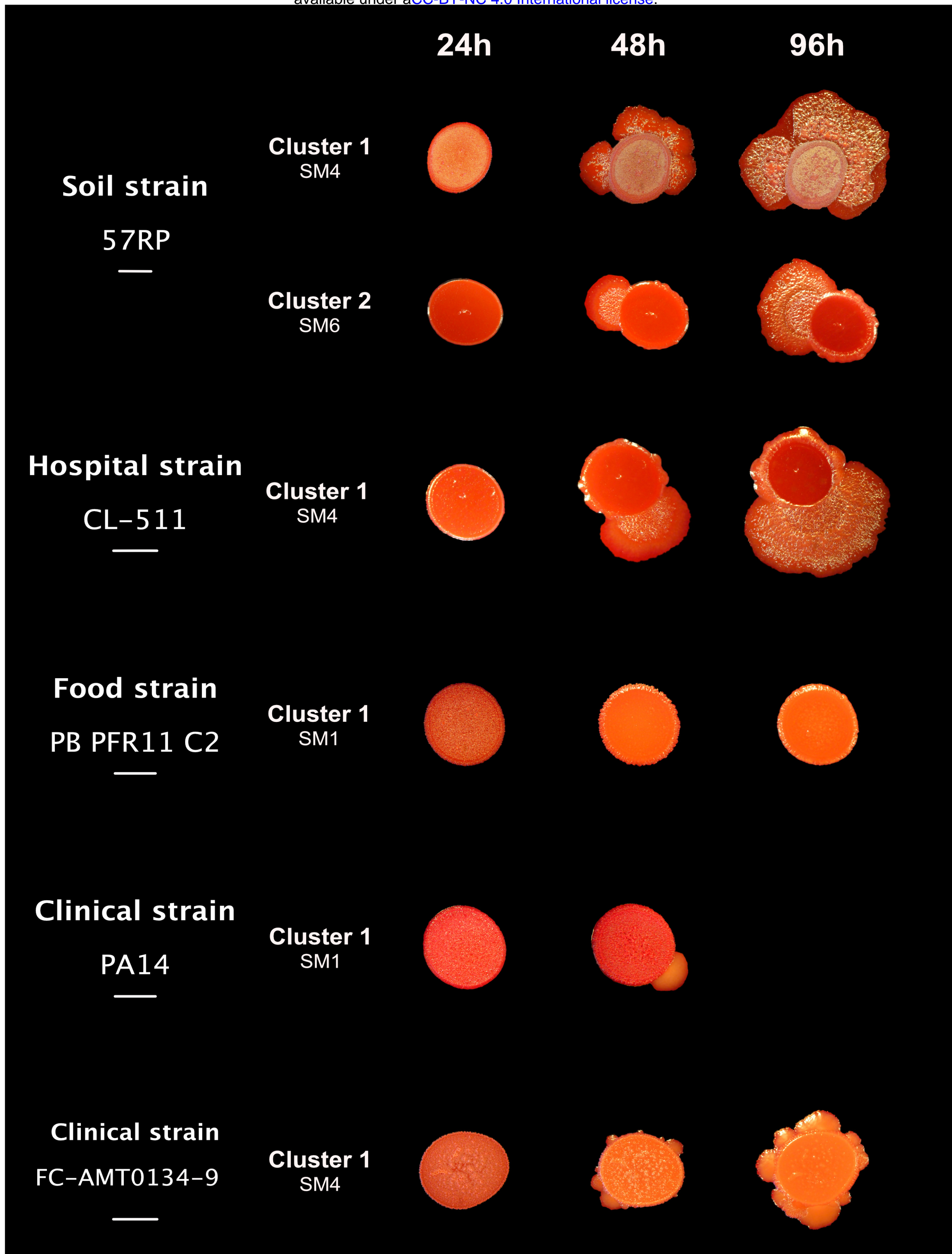

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Parental strain phenotype

SCV phenotype / Cluster 1 SMs

stable acquired mutation