AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

Pseudomonas aeruginosa Strains from Both Clinical and Environmental Origins Readily Adopt a Stable Small-Colony-Variant Phenotype Resulting from Single Mutations in c-di-GMP Pathways

Alison Besse,[a] Marie-Christine Groleau,[a] Mylène Trottier,[a] Antony T. Vincent,[b,c] Eric Déziel[a]

[a]Centre Armand-Frappier Santé Biotechnologie, Institut National de la Recherche Scientifique (INRS), Laval, Québec, Canada
[b]Département des Sciences Animales, Faculté des Sciences de l'Agriculture et de l'Alimentation, Université Laval, Québec City, Quebec, Canada
[c]Institut de Biologie Intégrative et des Systèmes, Université Laval, Québec City, Quebec, Canada

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

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

KEYWORDS RSCV, biofilms, microbial adaptation, pellicle, phenotypic variation

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

P. aeruginosa expresses a broad range of virulence determinants that counteract host immunity and promote survival (7). One of these factors is the ability to form biofilms. These organized communities contribute greatly to evasion of host immunity and antimicrobial treatments. For instance, the biofilm matrix delays the penetration of

Editor Michael Y. Galperin, NCBI, NLM, National Institutes of Health

Address correspondence to Eric Déziel, eric.deziel@inrs.ca.

The authors declare no conflict of interest.

Received 31 May 2022
Accepted 22 August 2022
Published 14 September 2022

antibiotics and host defense effectors (8–10). *P. aeruginosa* typically persists in the lungs of CF individuals as a biofilm (11, 12).

The emergence of a subpopulation of small-colony variants (SCVs) is a frequently observed feature of *P. aeruginosa* isolates from CF lung biofilms (13, 14). SCVs are characterized by circular opaque dwarf colonies with a diameter about 3 times smaller than that of wild-type (WT) colonies (14–17). Shortly after their first report, we proposed that SCVs were phenotypic variants (18). Several studies suggest that phenotypic switching could be regulated by a reversible adaptation mechanism, phase variation (18, 19), traditionally defined as a high-frequency on/off switch between phenotypes that operates in a heritable and reversible manner (20–22). Indeed, SCVs spontaneously revert to the WT-like morphotype (15, 16, 18, 23). And yet, recent studies have reported stable genetic mutations in *P. aeruginosa* leading to the SCV phenotype in *in vitro*-grown biofilms and an animal model of PA14 infection (14, 24, 25). The SCV phenotype is typically caused by mutations in genes involved in the metabolism of the intracellular second messenger c-di-GMP (14, 26). Among them, mutations in the Wsp (wrinkly spreader [WS]) pathway are the most frequently reported (14, 24, 27). The Wsp pathway is a chemosensory system resulting in activation of the diguanylate cyclase (DGC) WspR in response to surface sensing, which regulates the c-di-GMP pool, along with other DGCs (synthesis of c-di-GMP) and phosphodiesterases (PDEs; degradation of c-di-GMP) in *P. aeruginosa* (28–31).

c-di-GMP is largely involved in regulation of the phenotypic properties associated with SCVs, through binding to specific receptors. For instance, while overproduction of exopolysaccharides (EPS) (Pel and Psl) (14, 32) and a motility deficiency, notably flagellar, have been described for SCVs (16, 18, 33), high c-di-GMP levels activate the expression of the *pel* operon, leading to production of the EPS Pel, and repress flagellar motility (34–36). *P. aeruginosa* SCVs exhibit several other specific properties, such as cell surface hyperpiliation and adherence to abiotic surfaces (16, 18, 37). These properties promote biofilm formation (38). Additionally, SCVs exhibit autoaggregative properties (16, 37).

It is striking that SCVs have mostly been isolated from infected hosts, essentially CF individuals, or by extension from laboratory cultivation of strains sampled from infected hosts (13). For instance, several studies have recovered SCVs from lung, sputum, or deep throat swabs of CF individuals (12, 16, 17, 39). CF is not the only pathology associated with the emergence of *P. aeruginosa* SCVs. These variants have also been isolated from urine, fecal, endotracheal secretion, and pleural effusion samples from patients suffering from meningioma, anoxic encephalopathy, hepatocellular carcinoma, lung carcinoma, or grave asphyxia neonatorum (40). In addition to having been isolated from infected hosts, SCVs have also been generated under *in vivo* laboratory conditions. For instance, SCVs have been obtained *in vivo* from *P. aeruginosa* strains during infections in burn wound porcine models and murine models (24, 41). In the latter study, the authors clearly showed that SCVs that arose in the context of infection were due to stable genetic mutations in their genomes (24).

Intriguingly, 20 years ago, we reported one of the first identifications of *P. aeruginosa* SCVs that quickly emerged when a soil isolate was grown on a non-aqueous-phase liquid, hexadecane, as the sole substrate (18). The SCV morphotype of strain 57RP predominates when biofilm growth conditions are preferable and displays features shared with clinical SCVs: high adherence, efficient biofilm formation, hyperpiliation, and reduced motility (18). To our knowledge, that study is the only one reporting SCVs for an environmental *P. aeruginosa* isolate. However, the genetic cause leading to SCV emergence in the environmental context remains elusive. SCVs generated *in vitro* from *P. aeruginosa* strains PAO1 and PA14 showed stable mutations, but these strains, although prototypical, are still of clinical origin (25, 37).

Since most SCVs have until now been isolated from clinical samples, it remains unclear how widespread the ability of *P. aeruginosa* strains to develop this phenotype is and what the genetic mechanism(s) behind the emergence of SCVs are in regard to the origin of the isolate: are they exploiting phase variation or selecting adaptive mutants? Here, we investigated the ability of *P. aeruginosa* isolates from various environmental

origins to spontaneously adopt, under laboratory culture conditions, an SCV-like smaller colony morphotype readily distinguishable from that of their ancestral parent. We tested 22 *P. aeruginosa* strains from four different categories of environments: soil, food, hospital water systems, and clinical, and found that all the *P. aeruginosa* strains had the ability to adopt the SCV phenotype, regardless of their origin. Whole-genome sequencing was performed on SCVs from two strains isolated from distinct environments to investigate the potential genetic causes responsible for the SCV phenotypes. We found that mutations affecting c-di-GMP signaling pathways were responsible for SCV emergence in clinical and environmental strains.

RESULTS

The ability to form SCV-like morphotype colonies is a conserved feature of *Pseudomonas aeruginosa*. Culture conditions promoting biofilm formation select for SCVs of *P. aeruginosa* (16, 18, 37). To broadly investigate the ability of *P. aeruginosa* to adopt an SCV-like morphotype, we cultured 22 isolates from various origins in static liquid medium for 65 h and then spread them onto tryptic soy 2% agar (TS-agar 2%) plates to obtain isolated colonies. Six strains were from food samples (meat and fish from markets), six from clinical samples (five from CF patients and the prototypic clinical strain PA14 from a burn patient), five from petroleum oil-contaminated soil, and five from hospital sinks (drain, splash area, and tap) (Table 1). To cover the variety of temperatures relevant to these various habitats, the cultures were incubated in a range of temperatures from 30 to 40°C. At the outset, none of the strains displayed an SCV phenotype, but after 65 h of incubation, all isolates had diversified into a range of colony morphotypes, including small colonies that appeared typical of SCVs (Fig. 1 shows colonies of selected strains from each origin). Small colonies emerged in the cultures incubated at all temperatures tested (data not shown).

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

Isolated SCV-like morphotype colonies belong to two distinct clusters. By taking a closer look at the SCV-like morphotypes that emerged, we observed that their sizes (Table 1) and overall appearance (Fig. 1) differed. Some colonies were denser, with well-defined round edges, and others were more translucent, with undefined edges (Fig. 1). We then asked whether these different types of SCV-like morphotypes were indeed *bona fide* SCVs and if a distinction could be made between them. We focused on five strains from different origins (Table 1, strains indicated by boldface) and isolated the various morphotypically distinct small colonies (small morphotypes [SMs]) produced by each following static incubation and plating. Besides their sizes, we looked at several phenotypes typically associated with SCVs, including defective swimming motility, biofilm formation and production of EPS, cell aggregation, and production of c-di-GMP. Because cell aggregation induces the production of pyoverdine, the fluorescent siderophore of *P. aeruginosa*, while loss of the EPS-coding genes *pel* and *psl* leads to inhibition of pyoverdine production (42), we used the production of pyoverdine as an indirect measurement of cell aggregation and EPS production. We compiled the phenotypical data for each distinct SM (Table S1) and performed a principal coordinate analysis (PCoA) based on colony size, autoaggregation properties (pyoverdine production), ability to perform swimming motility, timing of biofilm formation, and total biomass of biofilms. In the PCoA, all variables were considered equally so as to cluster SCVs in significant groups based on their phenotypic profiles and better understand which SMs were close to each other and could be part of the same clusters. We found that the various distinct SMs generated by the five parental strains clustered in two separate groups (clusters 1 and clusters 2) (Fig. 2). Members of both clusters for the SMs of soil strain 57RP, the sink hospital strain CL-511, the food strain PB PFR11 C2, and the clinical strain FC-AMT0134-9 had phenotypical features that distinguished them from their parental strains (Fig. 2). Cluster 2 of strain PA14

TABLE 1 Colony diameters and prevalences of *P. aeruginosa* parental isolates and their static liquid culture-evolved small morphotypes

Source, strain[a]	Morphotype[b]	Avg colony diam (mm)[c]	Reference	Proportion of SM ± SD (%)
Clinical				
FC-AMT 0102-8	Parental isolate	1.57	76	
	SCV-like morphotypes	0.65 ± 0.09		8.14 ± 4.47
FC-AMT 0127-13	Parental isolate	2.24	76	
	SCV-like morphotypes	0.63 ± 0.15		99.2 ± 0.41
FC-AMT 0134-9	Parental isolate	4.21	76	
	SCV-like morphotypes	0.83 ± 0.26		6.91 ± 5.95
FC-AMT 0127-2	Parental isolate	2.19	76	
	SCV-like morphotypes	0.73 ± 0.18		19.3 ± 8.66
FC-AMT 0166-22	Parental isolate	2.27	76	
	SCV-like morphotypes	0.74 ± 0.22		6.41 ± 3.36
ED14/PA14	Parental isolate	3.16	77	
	SCV-like morphotypes	1.24 ± 0.14		44.4 ± 9.62
Food				
ABO VB50 C1	Parental isolate	4.50	78	
	SCV-like morphotypes	0.63 ± 0.39		6.44 ± 4.06
BG VB5 C2	Parental isolate	4.53	78	
	SCV-like morphotypes	1.12 ± 0.17		18.7 ± 5.63
PB PFR11 C2	Parental isolate	2.96	78	
	SCV-like morphotypes	1.17 ± 0.24		17.4 ± 7.98
ABO PF5 C1	Parental isolate	2.38	78	
	SCV-like morphotypes	0.84 ± 0.23		5.63 ± 0.20
BG VB11 C1	Parental isolate	2.28	78	
	SCV-like morphotypes	0.93 ± 0.17		4.87 ± 1.87
ADJ VB12 C1	Parental isolate	2.30	78	
	SCV-like morphotypes	0.91 ± 0.27		7.38 ± 3.22
Soil				
19SJV	Parental isolate	3.55	79	
	SCV-like morphotypes	1.01 ± 0.32		25.9 ± 10.6
34JR	Parental isolate	7.20	79	
	SCV-like morphotypes	1.78 ± 1.08		4.51 ± 1.33
57RP	Parental isolate	2.61	79	
	SCV-like morphotypes	1.07 ± 0.24		18.7 ± 8.79
18G	Parental isolate	10.14	79	
	SCV-like morphotypes	1.76 ± 1.45		11.1 ± 7.69
PG201	Parental isolate	6.08	80	
	SCV-like morphotypes	1.64 ± 0.86		20.4 ± 14.9
Hospital sink				
CL-511	Parental isolate	7.97	81	
	SCV-like morphotypes	1.56 ± 0.45		50.5 ± 29.2
CL-542a	Parental isolate	2.47	81	
	SCV-like morphotypes	0.95 ± 0.22		20.1 ± 5.29
CL-534a	Parental isolate	2.52	81	
	SCV-like morphotypes	0.72 ± 0.33		7.58 ± 2.29
CL-547b	Parental isolate	3.32	81	
	SCV-like morphotypes	0.97 ± 0.41		6.72 ± 0.73
PAO303	Parental isolate	3.63	82	
	SCV-like morphotypes	1.00 ± 0.55		5.29 ± 2.09

[a]Strains in boldface were selected for further phenotypic characterization.
[b]Colonies were considered to be SCV-like morphotype when their diameter was at least half that of the parental isolate.
[c]Average diameters of the small colonies.

contained only one isolated SM, but we believe that this is only the result of lower abundance of this form when sampling was performed. These results indicate that two distinct phenotypic types of SCV-like morphotypes emerged under our culture conditions.

SMs from cluster 1 are typical SCVs with a reversible state. SMs belonging to cluster 1 of each strain shared some common features: reduced swimming motility and/or promoted biofilm formation and/or enhanced autoaggregation properties (pyoverdine production) compared with their parental strain (Table S1 and Fig. S1 in

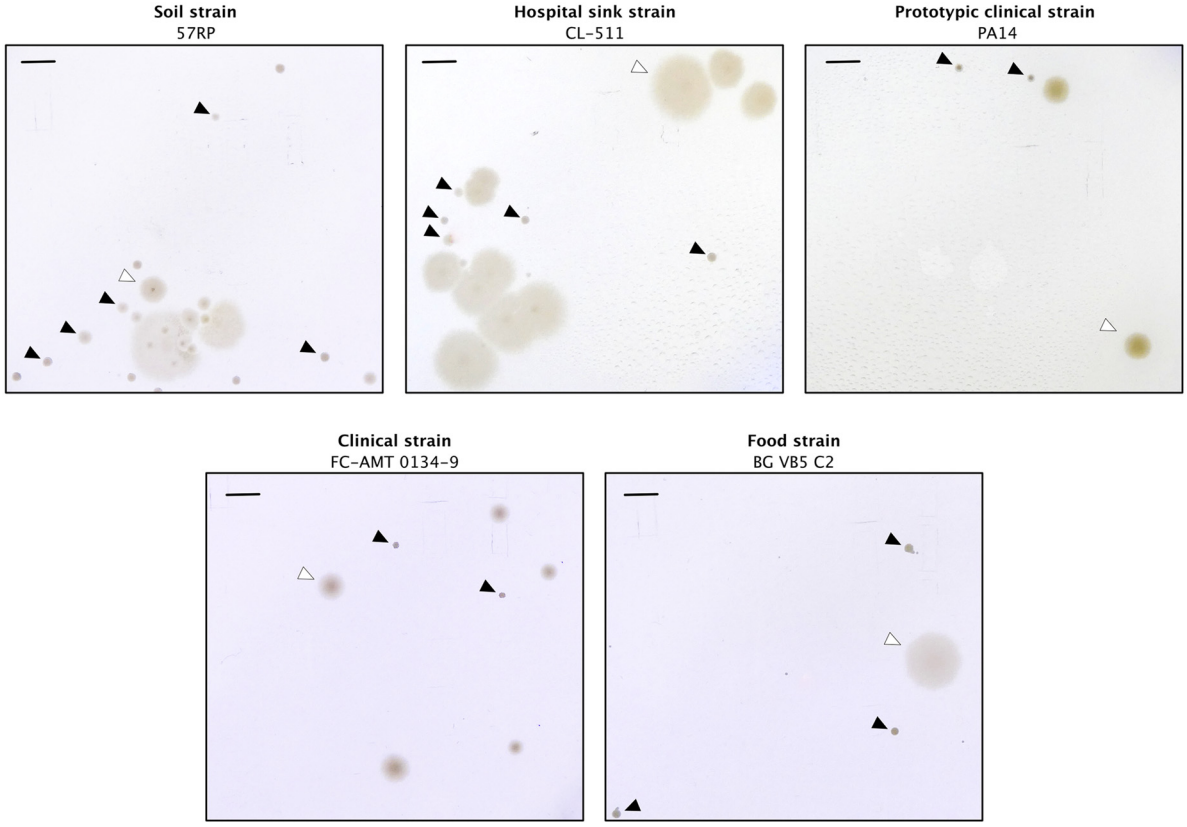

FIG 1 Small colonies of *Pseudomonas aeruginosa* emerged in static cultures from strains isolated from various origins. Parental strains were inoculated under static liquid conditions in TSB for 65 h and spread onto TS-agar 2% plates. Black arrowheads indicate smaller colonies. White arrowheads indicate parent-like colonies. Scale bars represent 5 mm.

the supplemental material). These features are typical of SCVs described in the literature. Since these phenotypes are regulated by c-di-GMP, we assessed intracellular c-di-GMP levels in selected SMs of clusters 1. As expected, higher c-di-GMP levels were measured in cluster 1 SMs than in their parental counterparts, again indicating that cluster 1 SMs were indeed typical SCVs (Fig. 3). In addition to the quantitative PCoA data, we looked at the rugosity of SM colonies, a qualitative phenotype traditionally associated with SCVs. While cluster 1 SM colonies displayed a very distinctive rugose surface compared with their parental counterparts, the appearance of rugosity was diverse among the strains (Fig. 4). In conclusion, phenotypic characterization confirmed that SMs belonging to cluster 1 were typical SCVs.

Finally, we observed the emergence of spontaneous reversion to a larger, parental-like phenotype, a property typically associated with phase variation. As stated above, on agar plates, reversion to the parental-like morphotype was observed after 48 h of incubation at 30°C for SMs belonging to clusters 1 (Fig. 5). Reversion was revealed as an outgrowth from the original colony, but sometimes only by a change in the appearance of the colony surface, as seen for instance with isolate PB PFR11 C2 (Fig. 5). This reversibility suggested that SCVs could arise from a phase variation process.

Cluster 1 SCVs harbor mutations in c-di-GMP pathways. To investigate whether SCVs could arise from phase variation, we performed whole-genome sequencing of strain PA14 and 57RP SCVs obtained from independent experiments of 65 h of static cultivation of the parental strains. The genomes of the parental strains were used as the references for the search for potential mutations in the SCVs' genomes. Mutations were found in all SCVs (Table 2). Interestingly, they were exclusively detected in genes involved in c-di-GMP metabolism. SCVs randomly selected from the first experiment with the parental strain PA14 carried missense single-nucleotide polymorphism (SNP)

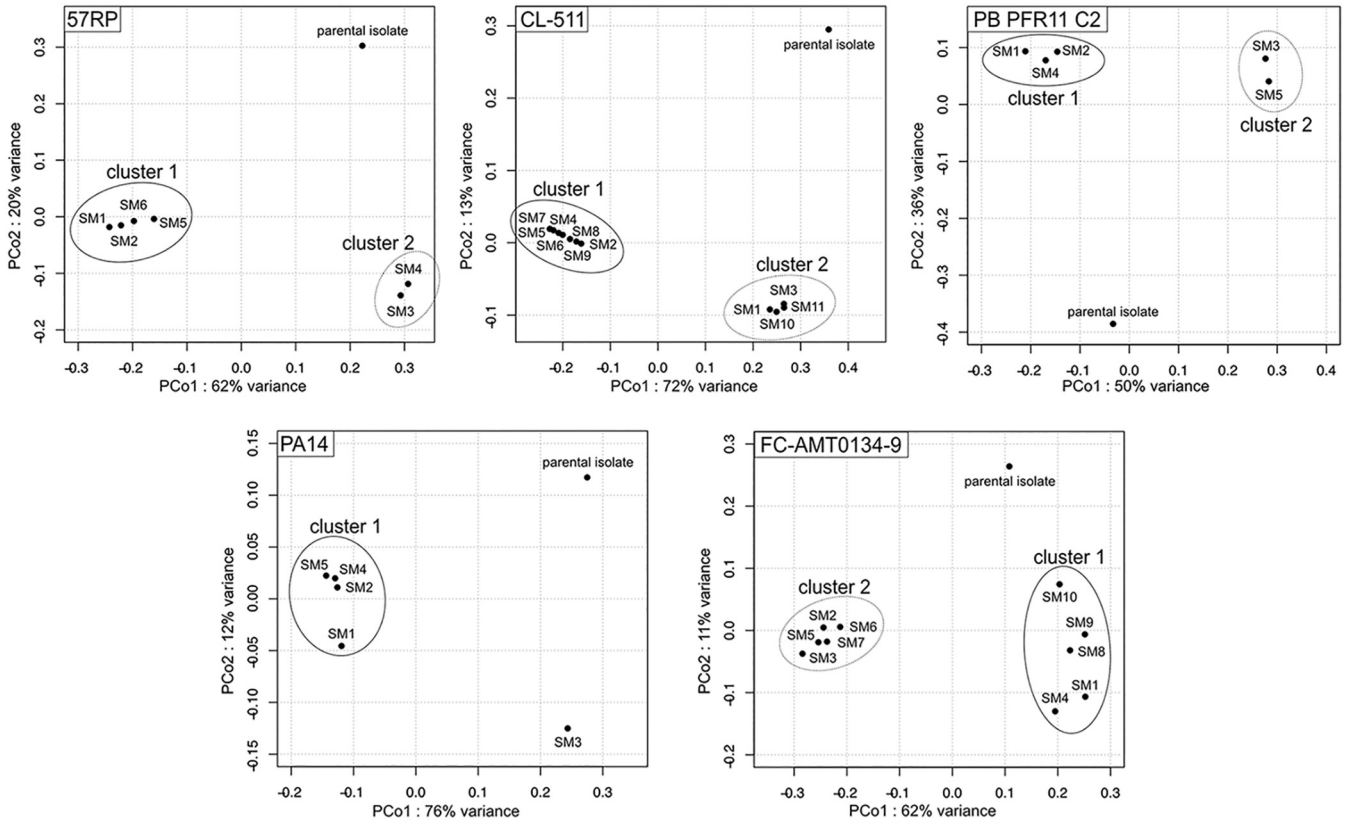

FIG 2 Small colonies isolated from static cultures clustered in two separate groups according to their phenotypic features. PCoA analysis was performed with a matrix composed of data obtained from the phenotypic analyses (swimming, biofilm formation, and pyoverdine production) for the parental strains and distinct small colonies isolated from static cultures with a diameter at least two times smaller than that of the parental strains (Table S1). All variables were considered equally to cluster colonies in significant groups based on their phenotypic profiles. Each point represents a small colony isolated from a static culture and has a name code composed of SM, standing for small morphotype, and an arbitrary number attributed during the isolation of the colonies. The identification of statistically distinctive clusters was performed using simprof tests and hclust.

mutations in the *yfiN* gene, while SCVs obtained from the second and third experiment had mutations in the *wsp* cluster, specifically in the *wspA* and *wspF* genes (Table 2). The mutations in the *wsp* cluster were SNPs resulting in a missense or stop codon mutation, except for one variant that showed a single base deletion leading to a frameshift. SCVs obtained from 57RP carried mutations exclusively in the *wspA* gene; specifically, an in-frame 42-bp deletion (amino acids [aa] 285 to 298 [Δ285–298]) was present in 12 sequenced SCVs out of 13 total (Table 2). The other sequenced 57RP SCV also carried a mutation in *wspA*, but it was a missense SNP leading to the replacement of a proline residue by a serine residue, potentially also resulting in modulation of WspA activity (Table 2). These mutations in PA14 and 57RP SCV genomes were likely responsible for the increased c-di-GMP levels we measured (Fig. 3). These results indicated that SCV emergence was largely due to mutations resulting in increased c-di-GMP levels. On the other hand, transposon mutants of *wspR* or *yfiN* in PA14, resulting in the inactivation of DGC WspR or YfiN, did not affect the rate of SCV emergence, suggesting that this phenomenon was regulated by interchangeable DGCs (Fig. S2). And yet, the genetic cause of the SCV phenotype remained elusive: did it always arise from stable mutations or was it a consequence of reversible mutations, accounting for the reversion described above?

Cluster 1 SCV emergence is due to stable mutations, and a second mutation is responsible for reversions. Despite the presence of mutations in the SCVs, reversion was still systematically observed on agar plates; this suggested that their emergence could be regulated by a phase variation mechanism. For each strain tested, reversions of SCVs were obtained directly within the colony by extending the incubation time of

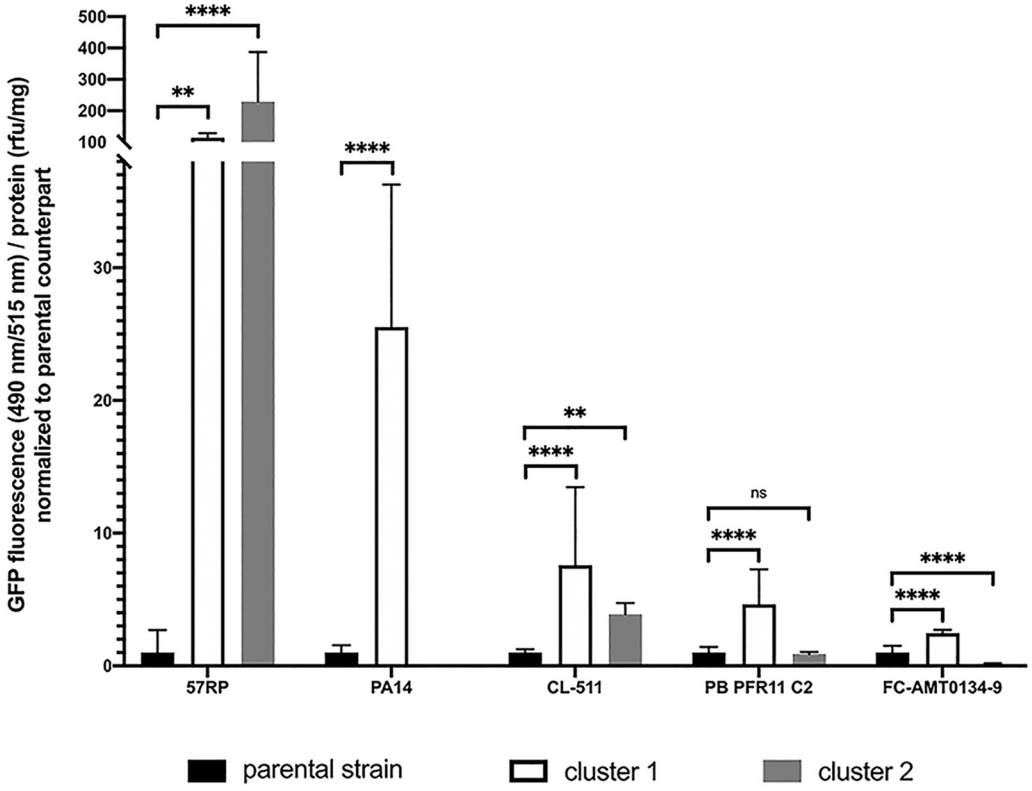

FIG 3 c-di-GMP production was altered for SMs from cluster 1 and 2 compared with their respective parental strains. c-di-GMP production was measured with the fluorescence-based biosensor pCdrA::*gfp*^{c} on washed O/N cultures. The values are means ± standard deviations (error bars) for selected transformed morphotypes belonging to each cluster. The transformed morphotypes were SM2 and SM6 (cluster 1) and SM4 (cluster 2) for strain 57RP, SM4 and SM5 (cluster 1) for strain PA14, SM8 and SM9 (cluster 1) and SM10 (cluster 2) for strain CL-511, SM1 and SM2 (cluster 1) and SM3 and SM6 (cluster 2) for strain PB PFRC11 2, and SM9 (cluster 1) and SM5 and SM7 (cluster 2) for strain FC-AMT0134-9. Three transformants of each SM were considered in the calculation of mean values and standard deviations. Asterisks represent the statistical significance of the results calculated by ordinary one-way analysis of variance (ANOVA). ****, $P \leq 0.0001$; **, $P \leq 0.01$; ns, not significant. Data were normalized between strains based on their parental strain. rfu, relative fluorescence units.

the plate (Fig. 5). However, the inoculation of SCVs under nonfavorable conditions, e.g., cultivation with agitation, did not enable detection of the emergence of revertants, regardless of the strain (data not shown). This result suggested that the mutations that occurred in PA14 and 57RP SCVs were rather stable. To determine if the SCV phenotype was due to a stable or a reversible genetic mutation, whole-genome sequencing was performed on reversion outgrowths of SCV PA14 (SM2) and 57RP (SM2) colonies (Fig. 5 and Table 2). In the PA14 SCV (SM2) outgrowth, a second SNP mutation was detected downstream from the first mutation in the same gene, *yfiN*, which resulted again in a missense codon. We supposed that this second mutation counterbalanced the effect of the first mutation and was responsible for the switch from SCV to another morphotype, probably by inactivating YfiN. In the 57RP SCV (SM2) outgrowth, a second mutation was also detected. However, this mutation was in a different gene, *wspR*, located functionally downstream from the mutated WspA. Thus, regardless of the origin of the strain, reversion was due to a second mutation, indicating that the SCV phenotype was due to the acquisition of a stable genetic mutation and reversion was not the result of phase variation (Fig. 5 and Table 2).

SMs from cluster 2 display phenotypical heterogeneity. Unlike cluster 1 SMs, SMs included in cluster 2 displayed interstrain diversity when considering the phenotypes used for the PCoA (Table S1 and Fig. S1). For instance, among cluster 2 SMs, the swimming motility was intermediate between those of the parental strain and cluster 1 SMs for strains 57RP and PB PFR11 C2 (Table S1 and Fig. S1A). However, for strains

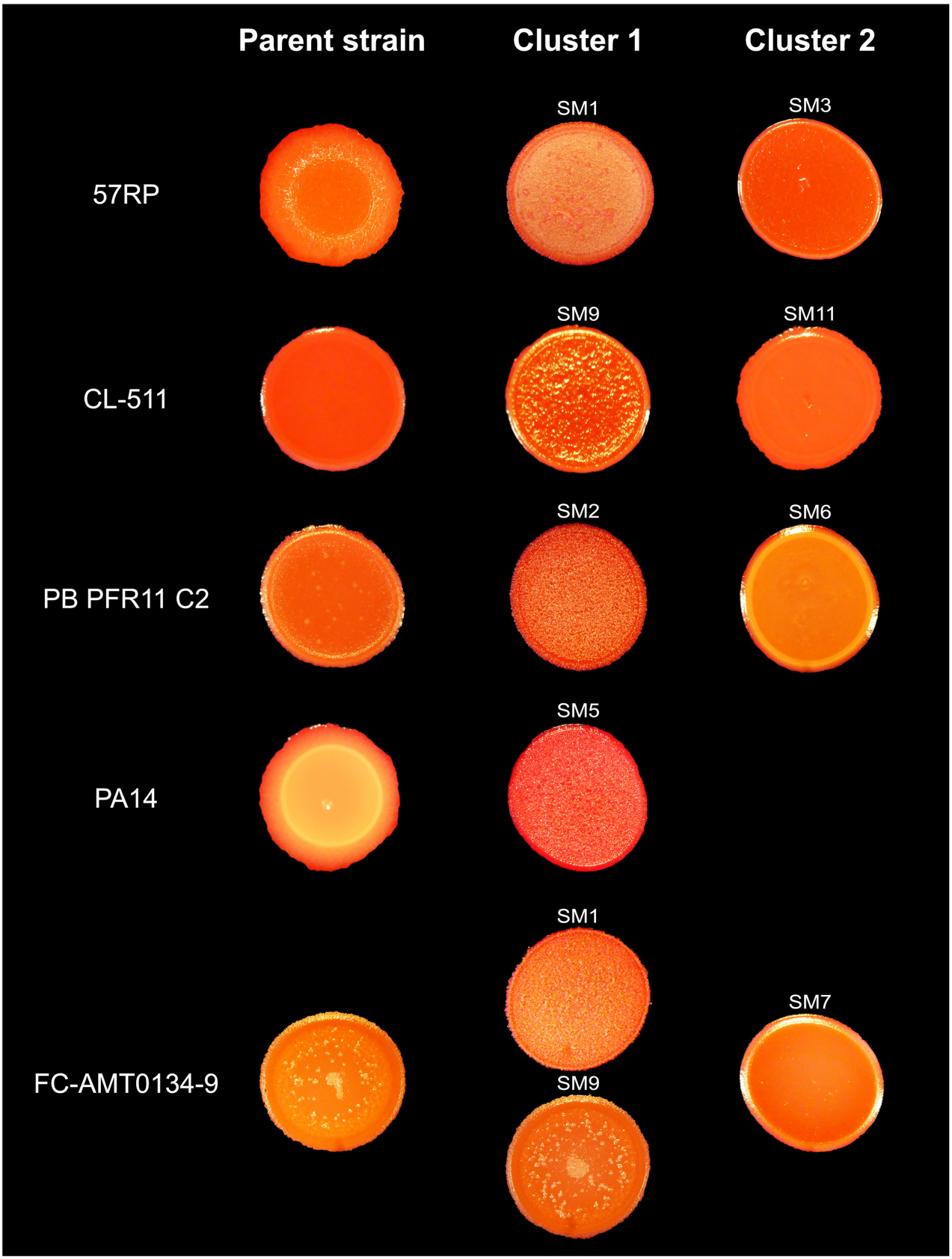

FIG 4 Appearance of colonies for the parental isolates and SMs from clusters 1 and clusters 2 on Congo red plates. The SM shown for each cluster is representative of all the SMs included in one cluster since they had a similar appearance. Plates were observed with a binocular Stemi DV4 microscope (Zeiss), and photos were taken with a DMC-ZS60 camera (Panasonic Lumix) after 24 h of incubation at 30°C.

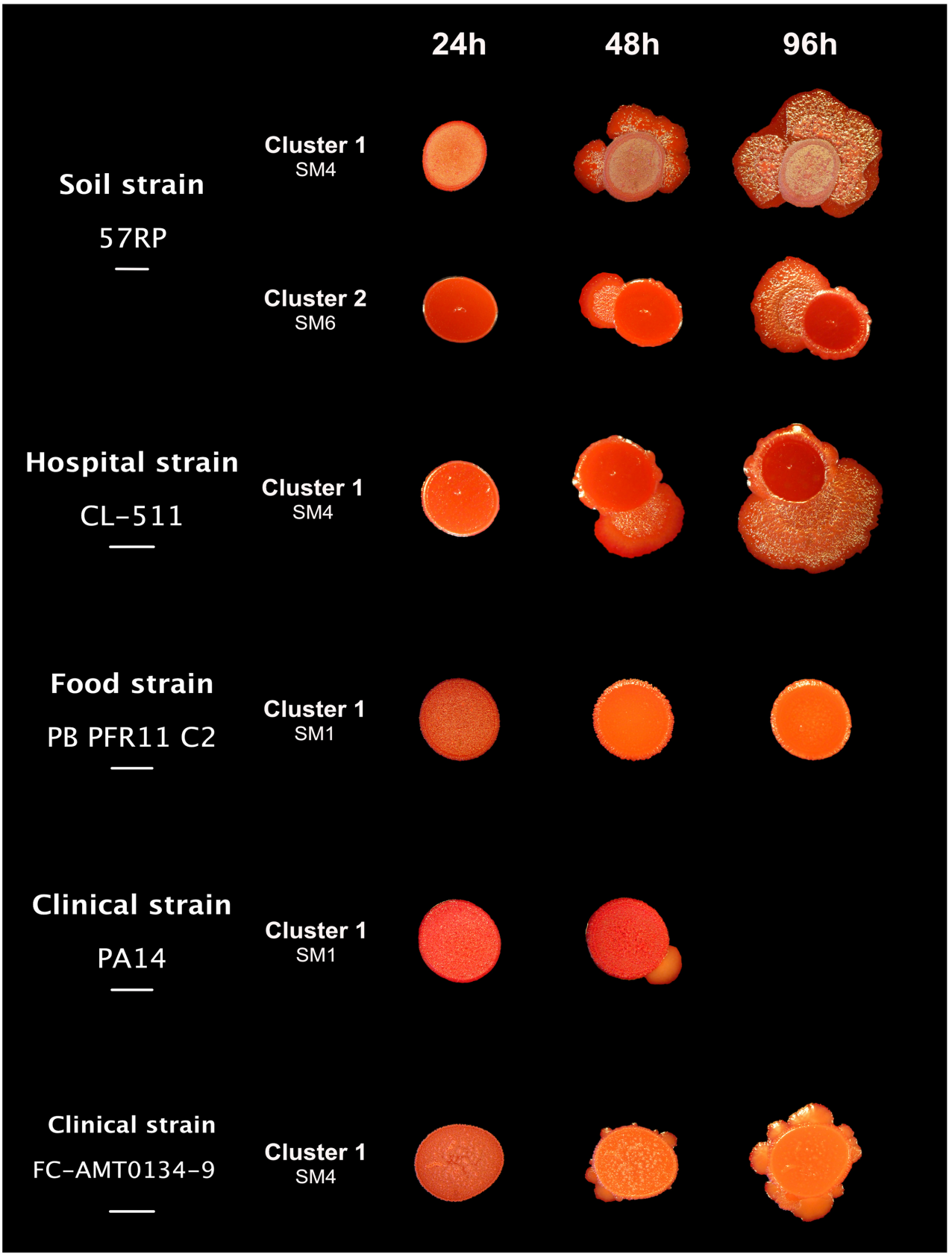

FIG 5 Reversion occurred on solid medium for specific morphotypes after 48 h of incubation. Amounts of 10 μL of cultures of parental strains or cluster-representative morphotypes (SMs) were dropped onto 0.1% Congo red TS-agar 2% plates. Plates were observed with a binocular Stemi DV4 microscope (Zeiss), and photos were taken with the camera DMC-ZS60 (Panasonic Lumix), after 24 h, 48 h, and 96 h of incubation at 30°C. Scale bars represent 5 mm.

TABLE 2 Mutations identified in strain 57RP and PA14 SCVs and their revertants

		Sequencing result compared to parental genome					
		SCV			Revertant		
Strain	Expt no.[a]	SCV no.	Gene	Mutation[b]	Name	Gene	Mutation[b]
57RP	1	1	*wspA*	del 285–298	Revertant-SCV no. 2	*wspA*	del 285–298
		2	*wspA*	del 285–298		*wspR*	Leu71ms
		3	*wspA*	del 285–298			
		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	Revertant-SCV no. 2	*yfiN*	Cys166ms
		2	*yfiN*	Cys166ms		*yfiN*	Asp304ms
		3	*yfiN*	Cys166ms			
	2	4	*wspF*	Val318fs			
	3	5	*wspF*	Gln297ns			
	4	6	*wspA*	Ala422ms			

[a]Each experiment was performed individually from a distinct inoculum of the parental strain.
[b]Deleted amino acid residues are indicated. del, deletion; ms, missense; fs, frameshift; ns, nonsense.
[c]Deletion was detected by PCR and the whole genome was not sequenced.

CL-511 and FC-AMT0134-9, the swimming motility was increased compared to those of both cluster 1 SMs and the parental strains (Table S1 and Fig. S1A). In addition to the PCoA data, c-di-GMP production in the cluster 2 SMs also varied depending on the parental strain: 57RP cluster 2 SMs showed higher levels of c-di-GMP than both the parental strain and cluster 1 SMs, but CL-511 cluster 2 SMs showed higher production of c-di-GMP only compared to the parental strain (Fig. 3). Also, cluster 2 SMs from the food strain PB PFR11 C2 showed similar levels of production of c-di-GMP and cluster 2 SMs from the clinical strain FC-AMT0134-9 even lower levels of production of c-di-GMP compared to their parental strains (Fig. 3). Thus, c-di-GMP levels were not a consistent driving feature for SMs belonging to cluster 2. The appearance of the colony surface of cluster 2 SMs was also distinct on Congo red plates, once again depending on the parental strain. Colonies of SM3 and SM4 from 57RP displayed a rugose surface, but it was less pronounced than for cluster 1 morphotypes (SM1, SM2, SM5, and SM6), in agreement with their reduced autoaggregative properties (Fig. 4 and Fig. S1D). For the other strains (PA14, PB PFR11 C2, CL-511, and FC-AMT0134-9), SMs from cluster 2 displayed a smoother surface on Congo red agar, closer to the appearance of the parental strain (Fig. 4). While cluster 2 SMs showed rapid emergence to reproducible phenotypes, reversion to a larger colonial morphotype akin to the WT after 96 h was only observed for 57RP cluster 2 SMs and not for the other strains (Fig. 5). All together, these results indicated that, apart from strain 57RP, SMs from cluster 2 did not exhibit most of the typically described features of SCVs.

DISCUSSION

The ability to switch to the SCV phenotype is a conserved feature among *P. aeruginosa* strains, regardless of their origin. SCVs have mostly been reported in the context of human infections, notably from CF individuals. A correlation between the emergence of *P. aeruginosa* SCVs and infection persistence in animal models was established, supporting the idea that the SCV phenotype confers a fitness advantage under chronic infection conditions (43–45). The switch toward the SCV morphotype

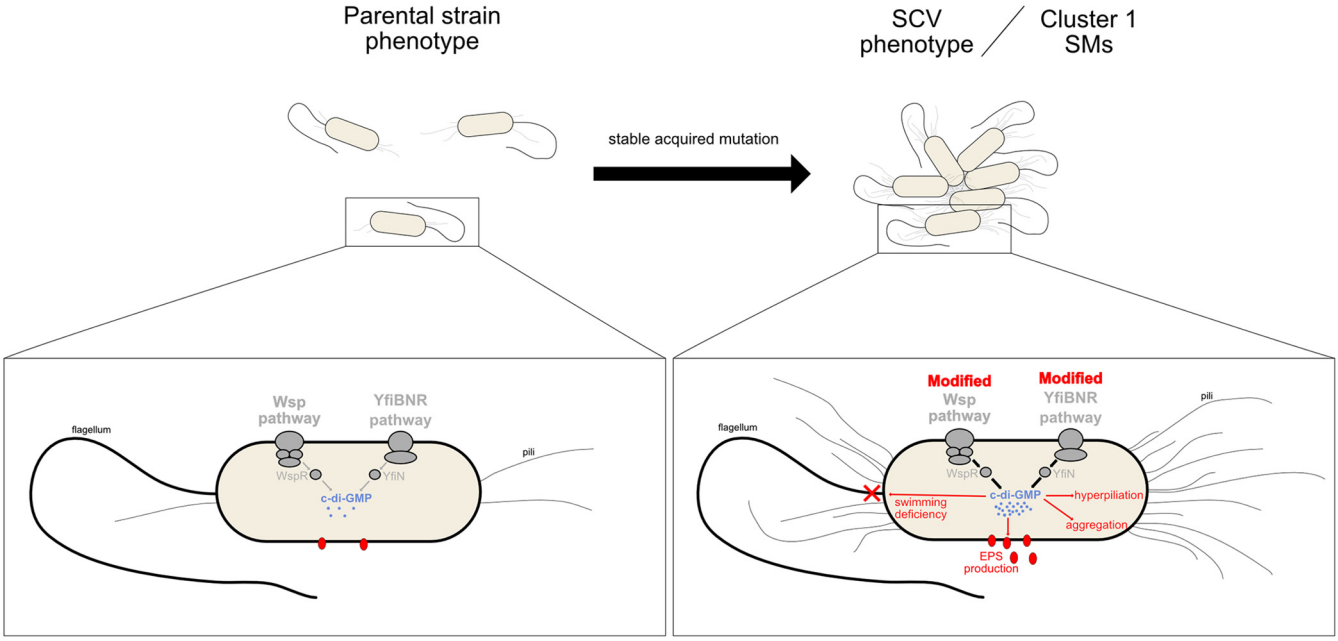

FIG 6 Schematic summary of SCV features compare to parental strain.

may represent an adaptation strategy for the hostile environment of the host by increasing resistance to host immunity and antimicrobial treatments (44, 46). However, the emergence of SCVs is not exclusively related to a clinical context. For instance, in 2001, Déziel et al. (18) reported the emergence of SCVs in laboratory cultures of a soil *P. aeruginosa* isolate. However, since then, apart from laboratory-grown prototypical strains *P. aeruginosa* PAO1 and PA14, both of clinical origin, no SCVs have been reported from a nonclinical context. Therefore, the question of prevalence remained open: is the ability to adopt an SCV phenotype mostly restricted to clinical isolates or the clinical context, from chronic infections, or not?

Here, we investigated the distribution of an SCV-based adaptative strategy in *P. aeruginosa* strains by screening 22 strains from diverse origins. Selective conditions were achieved by static cultivation, a culture condition that generates different microenvironments, as seen by the formation of a pellicle biofilm at the air-liquid interface. Plating of bacteria from static cultures of all 22 strains resulted in the formation of small colonies with sizes similar to those of SCVs described in other studies (16, 18). However, SCVs are not exclusively defined by the smaller size of their colonies. SCVs are also often identified based on the rugosity of the colony formed on Congo red agar plates, hence the alternate name RSCVs, for rugose small-colony variants (14, 24, 44). Nevertheless, rugosity is a subjective feature, and its description may vary according to the observer and culture conditions. Indeed, we have observed that the rugosity level varies between strains. This might be especially true for strains originating from diverse environments, as in the present study. Thus, we decided to take advantage of the various additional phenotypes described for SCVs to ascertain their identity (Fig. 6). To this end, we focused on five strains representing diverse environmental origins. Based on their phenotypic features, the small colonies obtained from each parental strain were clustered into two distinct groups. Small colonies classified in clusters 1 shared several interstrain phenotypic features, including reversion visible after 48 h. Based on known features, these small colonies can be defined as typical SCVs, validating that SCVs emerge from *P. aeruginosa* isolates from any origin. Thus, the ability to switch to the SCV phenotype appears to be an intrinsic feature of the species.

SCVs have always been isolated from biofilm-promoting conditions or from environments where biofilms thrive (16, 40, 41). SCVs are especially prone to adherence and biofilm formation (18, 37, 40). The attached mode of growth (biofilm) is a widespread

lifestyle in all types of environments (47–49). Biofilms are protective barriers for their bacterial components, and they increase tolerance to antimicrobials compared to that of free-living bacterial cells and enhance the ability to survive under extreme conditions, such as desiccation (50–52). Thus, one can easily conceive that the switch to the SCV phenotype confers a significant advantage for colonization of various ecological niches, accounting for the apparently conserved rapid switching to the SCV phenotype.

The SCV phenotype results from rapidly acquired stable genetic mutations in c-di-GMP systems. Intracellular c-di-GMP levels regulate all phenotypes associated with SCVs, including EPS production, motility, adherence, etc. (34–36). It is obvious that c-di-GMP thus plays a major role in the regulation of the SCV phenotype, but the role of c-di-GMP remains elusive in the mechanism of emergence. Several studies have reported that SCVs display mutations in genes involved in regulation of c-di-GMP levels, particularly genes included in the YfiBNR and Wsp pathways (14, 24, 44). In the SCVs that are frequently isolated from people with CF and are associated with persistent infection, mutations in *wsp* have also been reported while studying the genetic adaptation of *P. aeruginosa* to this specific environment (53, 54). These mutations lead to the activation of DGC and subsequent increase in c-di-GMP levels, either due to inactivation of the DGC repressor or constitutive activity of the chemosensory protein at the top of the signal transduction pathway. However, none of these studies were based on environmental strains, which is relevant since *P. aeruginosa* is naturally a saprophyte. Thus, the involvement of c-di-GMP in the regulation and/or emergence of the SCV phenotype from an environmental parental strain remains elusive. By sequencing the whole genomes of cluster 1 SCVs obtained from the prototypical clinical strain *P. aeruginosa* PA14 and the environmental strain *P. aeruginosa* 57RP, we wanted to determine if environmental strains can also switch to the SCV phenotype by using the same mechanism as PA14. We detected stable acquired mutations in all the PA14 SCV genomes we sequenced (14, 24). However, the previous reports reached their conclusions on the stability of the mutation in PA14 SCV by using genetic complementation and observing reversion to a parental-like morphotype (14, 24). Here, we present the first *in vitro* study that reaches a conclusion on the stability of mutations based on the whole-genome sequencing of revertants, highlighting a second spontaneous mutation responsible for the observed switching of morphotypes. Interestingly, Malone et al. isolated a clinical SCV with an acquired mutation from a CF patient and, 18 months later, isolated a planktonic (parental) phenotype from the same patient containing both the original mutation and a secondary loss-of-function mutation (32). Thus, phenotypic reversion occurring *in vitro* or *in vivo* is due to a secondary mutation, regardless of the origin of the strain. This indicates that the emergence of a secondary mutation and selection of revertants is not necessarily driven by the pressure of the infection-related clinical environment but is rather an intrinsic feature of *P. aeruginosa*.

In a previous report studying genetic evolution of PA14 upon infection in an animal model, Gloag et al. found that all PA14 SCVs displayed driver mutations in the Wsp pathway, mainly in *wspA* and a few in *wspF* (24). Wsp is a chemosensory pathway, activated by surface sensing from WspA and ultimately leading to the activation of the diguanylate cyclase WspR, which catalyzes the synthesis of c-di-GMP. This activation loop is regulated by the repressor WspF that acts to reset the system upon phosphorylation by WspE (28, 55). Here, PA14 SCVs from our standing culture conditions acquired mutations in the *wspA* and *wspF* genes of the Wsp pathway, but also in *yfiN*. The distinct membrane-integral DGC YfiN belongs to the YfiBNR pathway. YfiN activity is inversely controlled by the small periplasmic protein YfiR (repression) and the outer-membrane protein YfiB (44, 56). While a previous study has reported the importance of the *yfiBNR* operon in the emergence of SCVs in *P. aeruginosa* PAO1, our results confirm the importance of *yfiBNR* for SCV emergence in PA14 as well (32, 44). The interest is emphasized by the phylogenetic distance between strains PAO1 and PA14, which belong to two distinct phylogenetic groups of *P. aeruginosa* strains (57). In PAO1, engineered mutations of the gene encoding the DGC YfiN or its inhibitor YfiR led to an SCV phenotype (32, 44). Importantly, unlike in these previous studies on PAO1, the *yfiBNR*

mutation in the PA14 genome leading to an SCV phenotype arose spontaneously. To date, naturally acquired mutation(s) in *yfiN* have been reported only for two clinical SCVs isolated from CF patients (32). Like one of them, the mutation in PA14 was in the transmembrane helix domain, upstream from the DGC active domain (32). Surprisingly, this mutation resulted in an increased c-di-GMP level, maybe due to a distinct steric hindrance following the replacement of a cysteine residue by a serine.

Apart from studies of *P. aeruginosa* PAO1 and PA14, sometimes considered laboratory strains rather than clinical strains, this is the first report studying the mechanism of SCV phenotype emergence in *P. aeruginosa* environmental strains, such as 57RP. By sequencing the genomes of several independently evolved 57RP SCVs, we found that the same mutation occurred frequently in *wspA*. Interestingly, the exact same *wspA* Δ285–298 deletion mutation was also the most common mutation detected in PA14 SCVs upon infection of a murine chronic model (24). Several studies have reported mutations in this particular region (14, 58, 59), probably because this region may be hypermutable (24). And yet, it is striking that the deletion was the same in SCVs from PA14 and 57RP (24), especially since the SCV selection conditions were completely different. Since this deleted sequence is flanked by repeated inverted sequences, it could be a mobile element (24). However, we were not able to find this sequence at any other location in the parental-strain genomes, nor in revertant genomes, suggesting that this may not be a reversible deletion. Furthermore, the deletion was stable, since reversion was due to a second mutation in a downstream gene of the *wsp* operon. This deletion was proposed to lead to constitutive signaling and autoinduction of the Wsp pathway by alteration of methylation/demethylation of WspA, which would result in an increase in c-di-GMP production (24). While this is the first report of a Wsp mutation leading to SCV emergence in an environmental *P. aeruginosa* strain, it should be mentioned that a mutation in the Wsp pathway leading to an alternative phenotype was previously found in *Pseudomonas fluorescens* strain Pf0-1 (58, 60–62). Altogether, these results indicate that c-di-GMP plays a central role in SCV emergence in *P. aeruginosa*, in strains of both clinical and environmental origin. Interestingly, there are some striking parallels between the evolutionary origins of the wrinkly spreader (WS) phenotype of *P. fluorescens* and the SCV phenotype of *P. aeruginosa*, with both emerging during a standing incubation culture associated with the formation of a pellicle at the air-liquid interface. Indeed, *P. fluorescens* with the WS phenotype carries mutations in *wsp* and *yfi* (called *aws* in *P. fluorescens* strain SBW25) operons (63, 64). However, while showing some similarities in their respective genetic evolutive trajectories, the WS phenotype emerging in *P. fluorescens* and the SCV phenotype emerging in *P. aeruginosa* have clearly distinct features, such as the colony morphology (different sizes of the colony) and the genetic basis of the phenotype (overproduction of cellulose in *P. fluorescens* WS versus overexpression of *pel* and *psl* in *P. aeruginosa* SCV).

Interestingly, only one mutation was identified in the genomes of *P. aeruginosa* PA14 and 57RP SCVs. The sole other study that has also detected mutations having appeared spontaneously in *P. aeruginosa* PA14 SCV reported secondary mutations in the SCV genomes (24). Also, after a unique 65 h of incubation of the parental strain under static culture conditions, SCVs from both *P. aeruginosa* PA14 and 57RP represented 44.4% and 18.7% of the total population, respectively. This indicates that, regardless of the strain's origin, mutations in c-di-GMP pathways are selected *in vitro* to adapt to specific conditions and switch to the SCV phenotype (Fig. 6). Among all the c-di-GMP pathways known in *P. aeruginosa*, Wsp and YfiBNR seem to be preferred pathways involved in SCV emergence. However, although the increases in c-di-GMP resulting from alterations in Wsp and YfiBNR pathways are responsible for SCV emergence, the opposite is not true. Inactivation of the DGCs WspR and YfiN resulting in inability to produce c-di-GMP through this pathway did not affect the rate of emergence of SCVs. Thus, the regulation of SCV emergence through c-di-GMP mechanisms could be based on interchangeable DGCs, ready to take over upon inactivity of one of the pathways. Interestingly, in *P. fluorescens* SBW25, *wsp* mutations always arise first, then *aws* mutations (i.e., *yfi* in

P. aeruginosa) arise in strains lacking a Wsp system. Mutation of one or both of the *wsp* and *yfi* systems next leads to mutations in another c-di-GMP signaling system, *mwsA* (i.e., *morA* in *P. aeruginosa*) (63). However, while these results obtained in *P. fluorescens* open the discussion regarding the redundance of DGCs in *P. aeruginosa* during regulation of SCV emergence, the regulatory mechanisms might be distinct in the two species. Supporting this hypothesis, spontaneous mutations in *morA* have not yet been reported in *P. aeruginosa* SCVs.

SCVs could also emerge from a phase variation mechanism, undetectable under laboratory conditions. Phase variation is a common phenomenon among Gram-negative bacteria and is typical of bacteria thriving in heterogeneous ecological niches (21, 22, 65), notably *P. aeruginosa* (19). Unlike the acquisition of stable mutations, a phase variation mechanism represents a significant advantage for rapid adaptation to sudden changes in the environment (66, 67). Indeed, phase variation mechanisms lead to the emergence of a heterogeneous population in which the most suitable phenotype will multiply until the conditions fluctuate again and the selected phenotypes revert to another phenotype.

Although SCVs are due to stable genetic mutations under our experimental conditions, i.e., irreversible mutations, we cannot exclude the possibility that the adoption of the SCV phenotype could also rise from a reversible phase variation-regulated mechanism in natural habitats. Several reports support this hypothesis. First, phenotypes traditionally related to SCVs (motility and aggregation) are often regulated by phase variation mechanisms (21). In addition, reversible adaptation mechanisms are based on transitory DNA rearrangements (gene conversion, genomic inversion, and DNA recombination) and lead to variations in gene expression (20). Indeed, one recent study reports a large genomic inversion in *P. aeruginosa* SCVs (68). Finally, reversion of SCVs has been observed several times and could be due to phase variation instead of the emergence of a second mutation, but no whole-genome sequencing has been performed to investigate this (15, 16, 18). However, SCV reversion occurred toward a phenotype likely different from the parental morphotype (16, 23), suggesting that regulation is not necessarily an on/off switch on a particular locus and could be due to a secondary mutation in the genome.

Under our conditions, SCVs could have arisen from phase variation mechanisms, which was undetectable under our conditions with our techniques. Two elements could explain this limitation: (i) "reversible" SCVs were present but in undetectable quantities until they could be observed after sampling and agar spreading, or (ii) "reversible" SCVs were present but reverted to another morphotype when the samples were spread on agar plates. Indeed, various phenotypes were observed on agar plates after 65 h of standing incubation and were likely to have emerged in the static liquid culture, but they could also have emerged directly on the agar plate. To verify this hypothesis and verify that SCVs can also arise from a reversible mechanism, it would be interesting to follow the emergence of SCVs in the static culture using a detectable marker.

Small colonies are not necessarily SCVs or variants. During our experiments with static cultures, we observed several small-colony morphotypes. Based on our PCoA analysis, a proportion of them were clustered in two distinct groups (Fig. 2). Except for strain 57RP, the SMs from cluster 2 did not display clear reversion after 48 h on solid medium (data not shown). However, SMs from cluster 2 could still be able to revert under conditions outside the ones tested in our study. Thus, we wonder if cluster 2 SMs should be identified as variants based on our criteria.

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

The SCV phenotype has been linked to the persistence of *P. aeruginosa* in the context of infections in a human host, notably because of its increased resistance against

antimicrobials and host immunity. However, we have demonstrated here that strains isolated from soil, food, and hospital environments can also readily adopt an SCV phenotype. This indicates that the ability of *P. aeruginosa* to form SCVs is a conserved feature of this species and that the emergence of SCVs is not exclusively related to the pressure of the infection-related clinical environment. This is the first report of a high prevalence of SCVs among *P. aeruginosa* strains regardless of the origin of the isolates. The SCVs identified showed specific mutations in genes related to regulation of the intracellular levels of c-di-GMP. The Wsp and YfiBNR systems were the primary pathways used to increase c-di-GMP levels and switch to the SCV phenotype. The emergence of SCVs in various habitats allows *P. aeruginosa* to rapidly adapt and persist under diverse environmental conditions, accounting for its versatility and persistence. A deeper comprehension of the adaptation strategy used by *P. aeruginosa* could ultimately provide innovative strategies for the eradication of this opportunistic pathogen of public concern.

MATERIALS AND METHODS

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

Bradford protein assay. Due to the highly aggregative properties of SCVs, OD_{600} measurements were not appropriate to evaluate the growth of some of the isolated evolved morphotypes. Instead, the Bradford protein assay was used to quantify the concentration of total proteins in all our samples. Pellets from 1-mL amounts of cultures were resuspended in 1 mL 0.1 N NaOH and incubated for 1 h at 70°C. The protein concentrations of samples were measured according to the manufacturer's guidelines for the Bradford reagent (Alfa Aesar).

Phenotypic tests. Overnight (O/N) cultures of parental strains and their isolated morphotypes were grown at 30°C in an Infors incubator (Multitron pro) at 180 rpm in angled tubes. Since biofilm formation occurred in cultures, they were transferred to clean tubes to perform experiments or Bradford protein quantifications. Statistical analyses were achieved using ordinary one-way analysis of variance (ANOVA). Each phenotypic test was performed in technical triplicates.

Morphology on Congo red plates. A 1% Congo red solution in water (Fisher Scientific) was added to TS-agar 2% to a final concentration of 0.1%. Amounts of 10 μL of culture were spotted on the plates. Plates were incubated at 30°C and observed after 24 h, 48 h, and 96 h. Plates were observed with a binocular Stemi DV4 microscope (Zeiss), and photos were taken with a DMC-ZS60 camera (Panasonic Lumix).

Swimming motility tests. Swim plates (20 mM NH_4Cl, 12 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 0.5% Casamino Acids [CAA], 0.3% Bacto-Agar [BD], supplemented with 1 mM $MgSO_4$, 1 mM $CaCl_2$, and 11 mM dextrose) were prepared and dried for 15 min under the airflow of a biosafety cabinet. A volume of 2.5 μL of culture was inoculated into the agar. Plates were incubated for 20 h at 30°C. Swimming ability was assessed by measuring the area (mm^2) of the turbid circular zone using ImageJ. All experiments were performed in triplicates.

Biofilm formation. Microtiter (96-well) plates containing 1/10 TSB supplemented with 0.5% CAA were inoculated from a transferred O/N culture in order to obtain a starting concentration of 70 mM proteins. Each sample was inoculated into five different wells. The plates were incubated at 30°C without agitation. After 6 and 24 h, the plates were rinsed thoroughly with distilled water, and 200 μL of a 1% crystal violet solution was added to each well. After 15 min of incubation at room temperature, the plates were rinsed thoroughly with distilled water and the dye was solubilized in 300 μL of 30% acetic acid. The absorbance was measured at 595 nm with a microplate reader (Cytation 3; Biotek). Initiation of biofilm formation was calculated as the percentage of biofilm formed after 6 h of incubation compared with the total biofilm formed after 24 h of incubation. The total biomass of the biofilm was calculated as the amount of biofilm formed after 24 h, measured by crystal violet absorbance at 595 nm after 24 h of incubation.

Pyoverdine production. Overproduction of pyoverdine was previously noted as a feature of strain 57RP SCVs (18). We confirmed that an SCV from PA14 expressed high levels of fluorescence at the wavelength of pyoverdine emission, which likely accounts for its cell aggregation and EPS overproduction. An SCV isolated from a PA14 *pvdD* mutant (69), which was no longer able to produce pyoverdine, showed lower fluorescence levels, similar to those of parental colonies, confirming that (i) pyoverdine production was responsible for the fluorescence detected and (ii) the measured fluorescence was correlated with SCV aggregation properties (Fig. S3). To measure pyoverdine production, wells of black 96-well plates (Greiner) were filled with 200 μL of culture. Fluorescence was measured at wavelengths of 390 nm/530 nm (excitation/emission) using a multimode microplate reader (Cytation 3; Biotek).

c-di-GMP quantification. The intracellular levels of c-di-GMP were assessed with the fluorescence-based biosensor pCdrA::*gfp*^{c} (70, 71), acquired as Addgene plasmid no. 111614 (http://n2t.net/addgene:111614; Research Resource Identifier [RRID] Addgene_111614). Purified plasmids were transformed by electroporation into evolved morphotypes obtained from static cultures (72). Transformants were selected on TS-agar 2% supplemented with 100 μg/mL gentamicin. Three clones for each transformed morphotype were cultured in TSB supplemented with 100 μg/mL gentamicin. Cultures were washed twice in fresh TSB to get rid of a potential nonspecific fluorescence due to fluorescent pigments released by bacteria, as pyoverdine. Fluorescence was measured using a Cytation 3 microplate reader (BioTek) at 490 nm/515 nm (excitation/emission) in black 96-well plates (Greiner). The nontransformed strain was used as a control. Fluorescence from the control was subtracted from the fluorescence signal for the transformed strains.

PCoA analysis. Colonies identified as SMs compared with their parental isolate (see Results) were used to perform a principal coordinate analysis (PCoA). Statistical analyses were performed using RStudio software version 1.3.1093 (73), and normalized data are shown in Table S1. A Euclidean distance matrix was used to generate clustering of the bacterial isolates according to their phenotypical profiles. A similarity profile analysis (simprof) was performed to determine the number of significant clusters produced using hclust with the assumption of no *a priori* groups. Significant clusters were considered when at least two evolved morphotypes constituted them.

Sequencing and analysis. To analyze SCV genomes and compare them with the corresponding parental strain genomes, genomic DNA was extracted from 200 μL of O/N culture of colony variants in TSB using the EasyPure genomic DNA kit (Transgen Biotech) according to the manufacturer's protocol. Clonal DNA was sequenced on the Illumina NextSeq 2000 at the Microbial Genome Sequencing center (MiGS); 2 $\times$ 151-bp paired-end reads were trimmed and quality filtered using fastp version 0.23.2 (74). Filtered reads were assembled using Skesa through Shovill version 1.1.0 and annotated using prokka version 1.14.6 to be used as reference sequences (75). The reads were then used for variant calling using snippy version 4.6.0 (https://github.com/tseemann/snippy) with the default settings.

The presence of a 42-bp deletion in *wspA* for additional 57RP colony variants was confirmed by amplifying a 200-bp PCR fragment with primers F-CGGAGACTTCGCTCATGGT and R-AGAGCTCAAGGGCCTGGT. The detection of amplified products was performed using 2% agarose gel electrophoresis.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

ACKNOWLEDGMENTS

We thank Cynthia Bérubé for her help with the c-di-GMP biosensor preliminary experiments and Thays de Oliveira Pereira for critical reading of the manuscript.

This work was supported by grant number MOP-142466 from the Canadian Institutes of Health Research (CIHR). Alison Besse received a fellowship funded by the postdoctoral Calmette and Yersin grant from the Institut Pasteur.

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

A.B. and E.D. conceived the project, contributed to experimental design, and interpreted results. A.B., M.-C.G., and M.T. contributed to data acquisition. A.T.V. and A.B. analyzed sequencing. A.B. wrote the manuscript. M.-C.G. and E.D. reviewed and edited the manuscript.

REFERENCES

1. Bédard E, Prévost M, Déziel E. 2016. *Pseudomonas aeruginosa* in premise plumbing of large buildings. Microbiologyopen 5:937–956. https://doi.org/10.1002/mbo3.391.
2. Diggle SP, Whiteley M. 2020. Microbe profile. *Pseudomonas aeruginosa*: opportunistic pathogen and lab rat. Microbiology (Reading) 166:30–33. https://doi.org/10.1099/mic.0.000860.
3. Crone S, Vives-Flórez M, Kvich L, Saunders AM, Malone M, Nicolaisen MH, Martínez-García E, Rojas-Acosta C, Catalina Gomez-Puerto M, Calum H, Whiteley M, Kolter R, Bjarnsholt T. 2020. The environmental occurrence of *Pseudomonas aeruginosa*. APMIS 128:220–231. https://doi.org/10.1111/apm.13010.
4. Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Hoiby N, Molin S. 2012. Adaptation of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. Nat Rev Microbiol 10:841–851. https://doi.org/10.1038/nrmicro2907.
5. Malhotra S, Hayes D, Jr, Wozniak DJ. 2019. Cystic fibrosis and *Pseudomonas aeruginosa*: the host-microbe interface. Clin Microbiol Rev 32:e00138-18. https://doi.org/10.1128/CMR.00138-18.
6. López-Causapé C, Cabot G, Del Barrio-Tofiño E, Oliver A. 2018. The versatile mutational resistome of *Pseudomonas aeruginosa*. Front Microbiol 9:685. https://doi.org/10.3389/fmicb.2018.00685.
7. Gellatly SL, Hancock RE. 2013. *Pseudomonas aeruginosa:* new insights into pathogenesis and host defenses. Pathog Dis 67:159–173. https://doi.org/10.1111/2049-632X.12033.
8. Williams BJ, Dehnbostel J, Blackwell TS. 2010. Pseudomonas aeruginosa: host defence in lung diseases. Respirology 15:1037–1056. https://doi.org/10.1111/j.1440-1843.2010.01819.x.
9. Alhede M, Bjarnsholt T, Givskov M, Alhede M. 2014. *Pseudomonas aeruginosa* biofilms: mechanisms of immune evasion. Adv Appl Microbiol 86:1–40. https://doi.org/10.1016/B978-0-12-800262-9.00001-9.
10. Ciofu O, Tolker-Nielsen T. 2019. Tolerance and resistance of *Pseudomonas aeruginosa* biofilms to antimicrobial agents—how *P. aeruginosa* can escape antibiotics. Front Microbiol 10:913. https://doi.org/10.3389/fmicb.2019.00913.
11. Hoiby N, Ciofu O, Bjarnsholt T. 2010. *Pseudomonas aeruginosa* biofilms in cystic fibrosis. Future Microbiol 5:1663–1674. https://doi.org/10.2217/fmb.10.125.

12. Costerton JW, Stewart PS, Greenberg EP. 1999. Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322. https://doi.org/10.1126/science.284.5418.1318.
13. von Gotz F, Haussler S, Jordan D, Saravanamuthu SS, Wehmhoner D, Strussmann A, Lauber J, Attree I, Buer J, Tummler B, Steinmetz I. 2004. Expression analysis of a highly adherent and cytotoxic small colony variant of *Pseudomonas aeruginosa* isolated from a lung of a patient with cystic fibrosis. J Bacteriol 186:3837–3847. https://doi.org/10.1128/JB.186.12.3837-3847.2004.
14. Starkey M, Hickman JH, Ma L, Zhang N, De Long S, Hinz A, Palacios S, Manoil C, Kirisits MJ, Starner TD, Wozniak DJ, Harwood CS, Parsek MR. 2009. *Pseudomonas aeruginosa* rugose small-colony variants have adaptations that likely promote persistence in the cystic fibrosis lung. J Bacteriol 191:3492–3503. https://doi.org/10.1128/JB.00119-09.
15. Häussler S, Tümmler B, Weissbrodt H, Rohde M, Steinmetz I. 1999. Small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis. Clin Infect Dis 29:621–625. https://doi.org/10.1086/598644.
16. Häußler S, Ziegler I, Löttel A, Götz FV, Rohde M, Wehmhöhner D, Saravanamuthu S, Tümmler B, Steinmetz I. 2003. Highly adherent small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis lung infection. J Med Microbiol 52:295–301. https://doi.org/10.1099/jmm.0.05069-0.
17. Lozano C, Azcona-Gutiérrez JM, Van Bambeke F, Sáenz Y. 2018. Great phenotypic and genetic variation among successive chronic *Pseudomonas aeruginosa* from a cystic fibrosis patient. PLoS One 13:e0204167. https://doi.org/10.1371/journal.pone.0204167.
18. Déziel E, Comeau Y, Villemur R. 2001. Initiation of biofilm formation by *Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpiliated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. J Bacteriol 183:1195–1204. https://doi.org/10.1128/JB.183.4.1195-1204.2001.
19. Drenkard E, Ausubel FM. 2002. *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. Nature 416:740–743. https://doi.org/10.1038/416740a.
20. Villemur R, Déziel E. 2005. Phase variation and antigenic variation, p 277–322. *In* Mullany P (ed), The dynamic bacterial genome. Cambridge University Press, Cambridge, UK. https://doi.org/10.1017/CBO9780511541544.008.
21. van der Woude MW, Bäumler AJ. 2004. Phase and antigenic variation in bacteria. Clin Microbiol Rev 17:581–611. https://doi.org/10.1128/CMR.17.3.581-611.2004.
22. Henderson IR, Owen P, Nataro JP. 1999. Molecular switches—the ON and OFF of bacterial phase variation. Mol Microbiol 33:919–932. https://doi.org/10.1046/j.1365-2958.1999.01555.x.
23. Haussler S. 2004. Biofilm formation by the small colony variant phenotype of *Pseudomonas aeruginosa*. Environ Microbiol 6:546–551. https://doi.org/10.1111/j.1462-2920.2004.00618.x.
24. Gloag ES, Marshall CW, Snyder D, Lewin GR, Harris JS, Santos-Lopez A, Chaney SB, Whiteley M, Cooper VS, Wozniak DJ. 2019. *Pseudomonas aeruginosa* interstrain dynamics and selection of hyperbiofilm mutants during a chronic infection. mBio 10:e01698-19. https://doi.org/10.1128/mBio.01698-19.
25. Flynn KM, Dowell G, Johnson TM, Koestler BJ, Waters CM, Cooper VS. 2016. Evolution of ecological diversity in biofilms of *Pseudomonas aeruginosa* by altered cyclic diguanylate signaling. J Bacteriol 198:2608–2618. https://doi.org/10.1128/JB.00048-16.
26. Evans TJ. 2015. Small colony variants of *Pseudomonas aeruginosa* in chronic bacterial infection of the lung in cystic fibrosis. Future Microbiol 10:231–239. https://doi.org/10.2217/fmb.14.107.
27. Chua SL, Ding Y, Liu Y, Cai Z, Zhou J, Swarup S, Drautz-Moses DI, Schuster SC, Kjelleberg S, Givskov M, Yang L. 2016. Reactive oxygen species drive evolution of pro-biofilm variants in pathogens by modulating cyclic-di-GMP levels. Open Biol 6:160162. https://doi.org/10.1098/rsob.160162.
28. Valentini M, Filloux A. 2016. Biofilms and cyclic di-GMP (c-di-GMP) signaling: lessons from *Pseudomonas aeruginosa* and other bacteria. J Biol Chem 291:12547–12555. https://doi.org/10.1074/jbc.R115.711507.
29. Ha DG, Richman ME, O'Toole GA. 2014. Deletion mutant library for investigation of functional outputs of cyclic diguanylate metabolism in *Pseudomonas aeruginosa* PA14. Appl Environ Microbiol 80:3384–3393. https://doi.org/10.1128/AEM.00299-14.
30. Huangyutitham V, Güvener ZT, Harwood CS. 2013. Subcellular clustering of the phosphorylated WspR response regulator protein stimulates its diguanylate cyclase activity. mBio 4:e00242-13. https://doi.org/10.1128/mBio.00242-13.
31. Hickman JW, Tifrea DF, Harwood CS. 2005. A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. Proc Natl Acad Sci U S A 102:14422–14427. https://doi.org/10.1073/pnas.0507170102.
32. Malone JG, Jaeger T, Manfredi P, Dotsch A, Blanka A, Bos R, Cornelis GR, Haussler S, Jenal U. 2012. The YfiBNR signal transduction mechanism reveals novel targets for the evolution of persistent *Pseudomonas aeruginosa* in cystic fibrosis airways. PLoS Pathog 8:e1002760. https://doi.org/10.1371/journal.ppat.1002760.
33. Wei Q, Tarighi S, Dotsch A, Haussler S, Musken M, Wright VJ, Camara M, Williams P, Haenen S, Boerjan B, Bogaerts A, Vierstraete E, Verleyen P, Schoofs L, Willaert R, De Groote VN, Michiels J, Vercammen K, Crabbe A, Cornelis P. 2011. Phenotypic and genome-wide analysis of an antibiotic-resistant small colony variant (SCV) of *Pseudomonas aeruginosa*. PLoS One 6:e29276. https://doi.org/10.1371/journal.pone.0029276.
34. Baker AE, Diepold A, Kuchma SL, Scott JE, Ha DG, Orazi G, Armitage JP, O'Toole GA. 2016. PilZ domain protein FlgZ mediates cyclic di-GMP-dependent swarming motility control in *Pseudomonas aeruginosa*. J Bacteriol 198:1837–1846. https://doi.org/10.1128/JB.00196-16.
35. Hickman JW, Harwood CS. 2008. Identification of FleQ from *Pseudomonas aeruginosa* as a c-di-GMP-responsive transcription factor. Mol Microbiol 69:376–389. https://doi.org/10.1111/j.1365-2958.2008.06281.x.
36. Lee VT, Matewish JM, Kessler JL, Hyodo M, Hayakawa Y, Lory S. 2007. A cyclic-di-GMP receptor required for bacterial exopolysaccharide production. Mol Microbiol 65:1474–1484. https://doi.org/10.1111/j.1365-2958.2007.05879.x.
37. Kirisits MJ, Prost L, Starkey M, Parsek MR. 2005. Characterization of colony morphology variants isolated from *Pseudomonas aeruginosa* biofilms. Appl Environ Microbiol 71:4809–4821. https://doi.org/10.1128/AEM.71.8.4809-4821.2005.
38. Chiang P, Burrows LL. 2003. Biofilm formation by hyperpiliated mutants of *Pseudomonas aeruginosa*. J Bacteriol 185:2374–2378. https://doi.org/10.1128/JB.185.7.2374-2378.2003.
39. Schneider M, Muhlemann K, Droz S, Couzinet S, Casaulta C, Zimmerli S. 2008. Clinical characteristics associated with isolation of small-colony variants of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from respiratory secretions of patients with cystic fibrosis. J Clin Microbiol 46:1832–1834. https://doi.org/10.1128/JCM.00361-08.
40. Ikeno T, Fukuda K, Ogawa M, Honda M, Tanabe T, Taniguchi H. 2007. Small and rough colony *Pseudomonas aeruginosa* with elevated biofilm formation ability isolated in hospitalized patients. Microbiol Immunol 51:929–938. https://doi.org/10.1111/j.1348-0421.2007.tb03989.x.
41. Bayes HK, Ritchie N, Irvine S, Evans TJ. 2016. A murine model of early *Pseudomonas aeruginosa* lung disease with transition to chronic infection. Sci Rep 6:35838. https://doi.org/10.1038/srep35838.
42. Visaggio D, Pasqua M, Bonchi C, Kaever V, Visca P, Imperi F. 2015. Cell aggregation promotes pyoverdine-dependent iron uptake and virulence in *Pseudomonas aeruginosa*. Front Microbiol 6:902. https://doi.org/10.3389/fmicb.2015.00902.
43. Mulcahy H, O'Callaghan J, O'Grady EP, Maciá MD, Borrell N, Gómez C, Casey PG, Hill C, Adams C, Gahan CG, Oliver A, O'Gara F. 2008. *Pseudomonas aeruginosa* RsmA plays an important role during murine infection by influencing colonization, virulence, persistence, and pulmonary inflammation. Infect Immun 76:632–638. https://doi.org/10.1128/IAI.01132-07.
44. Malone JG, Jaeger T, Spangler C, Ritz D, Spang A, Arrieumerlou C, Kaever V, Landmann R, Jenal U. 2010. YfiBNR mediates cyclic di-GMP dependent small colony variant formation and persistence in *Pseudomonas aeruginosa*. PLoS Pathog 6:e1000804. https://doi.org/10.1371/journal.ppat.1000804.
45. Byrd MS, Pang B, Hong W, Waligora EA, Juneau RA, Armbruster CE, Weimer KE, Murrah K, Mann EE, Lu H, Sprinkle A, Parsek MR, Kock ND, Wozniak DJ, Swords WE. 2011. Direct evaluation of *Pseudomonas aeruginosa* biofilm mediators in a chronic infection model. Infect Immun 79:3087–3095. https://doi.org/10.1128/IAI.00057-11.
46. Malone JG. 2015. Role of small colony variants in persistence of *Pseudomonas aeruginosa* infections in cystic fibrosis lungs. Infect Drug Resist 8:237–247. https://doi.org/10.2147/IDR.S68214.
47. Chiellini C, Chioccioli S, Vassallo A, Mocali S, Miceli E, Fagorzi C, Bacci G, Coppini E, Fibbi D, Bianconi G, Canganella F, Fani R. 2019. Exploring the bacterial communities of Infernaccio Waterfalls: a phenotypic and molecular characterization of *Acinetobacter* and *Pseudomonas* strains living in a red epilithic biofilm. Diversity 11:175. https://doi.org/10.3390/d11100175.
48. Wingender J, Flemming HC. 2011. Biofilms in drinking water and their role as reservoir for pathogens. Int J Hyg Environ Health 214:417–423. https://doi.org/10.1016/j.ijheh.2011.05.009.
49. McBain AJ, Bartolo RG, Catrenich CE, Charbonneau D, Ledder RG, Rickard AH, Symmons SA, Gilbert P. 2003. Microbial characterization of biofilms in

domestic drains and the establishment of stable biofilm microcosms. Appl Environ Microbiol 69:177–185. https://doi.org/10.1128/AEM.69.1.177-185.2003.
50. Flemming HC, Wingender J, Szewzyk U, Steinberg P, Rice SA, Kjelleberg S. 2016. Biofilms: an emergent form of bacterial life. Nat Rev Microbiol 14:563–575. https://doi.org/10.1038/nrmicro.2016.94.
51. Lee K, Yoon SS. 2017. *Pseudomonas aeruginosa* biofilm, a programmed bacterial life for fitness. J Microbiol Biotechnol 27:1053–1064. https://doi.org/10.4014/jmb.1611.11056.
52. Soares A, Alexandre K, Etienne M. 2020. Tolerance and persistence of Pseudomonas aeruginosa in biofilms exposed to antibiotics: molecular mechanisms, antibiotic strategies and therapeutic perspectives. Front Microbiol 11:2057. https://doi.org/10.3389/fmicb.2020.02057.
53. Smith EE, Buckley DG, Wu Z, Saenphimmachak C, Hoffman LR, D'Argenio DA, Miller SI, Ramsey BW, Speert DP, Moskowitz SM, Burns JL, Kaul R, Olson MV. 2006. Genetic adaptation by *Pseudomonas aeruginosa* to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A 103:8487–8492. https://doi.org/10.1073/pnas.0602138103.
54. Marvig RL, Sommer LM, Molin S, Johansen HK. 2015. Convergent evolution and adaptation of Pseudomonas aeruginosa within patients with cystic fibrosis. Nat Genet 47:57–64. https://doi.org/10.1038/ng.3148.
55. Güvener ZT, Harwood CS. 2007. Subcellular location characteristics of the *Pseudomonas aeruginosa* GGDEF protein, WspR, indicate that it produces cyclic-di-GMP in response to growth on surfaces. Mol Microbiol 66:1459–1473. https://doi.org/10.1111/j.1365-2958.2007.06008.x.
56. Giardina G, Paiardini A, Fernicola S, Franceschini S, Rinaldo S, Stelitano V, Cutruzzolà F. 2013. Investigating the allosteric regulation of YfiN from *Pseudomonas aeruginosa*: clues from the structure of the catalytic domain. PLoS One 8:e81324. https://doi.org/10.1371/journal.pone.0081324.
57. Vincent AT, Freschi L, Jeukens J, Kukavica-Ibrulj I, Emond-Rheault JG, Leduc A, Boyle B, Jean-Pierre F, Groleau MC, Déziel E, Barbeau J, Charette SJ, Levesque RC. 2017. Genomic characterisation of environmental *Pseudomonas aeruginosa* isolated from dental unit waterlines revealed the insertion sequence ISPa11 as a chaotropic element. FEMS Microbiol Ecol 93:fix106. https://doi.org/10.1093/femsec/fix106.
58. Kim W, Levy SB, Foster KR. 2016. Rapid radiation in bacteria leads to a division of labour. Nat Commun 7:10508. https://doi.org/10.1038/ncomms10508.
59. Cooper VS, Staples RK, Traverse CC, Ellis CN. 2014. Parallel evolution of small colony variants in *Burkholderia cenocepacia* biofilms. Genomics 104:447–452. https://doi.org/10.1016/j.ygeno.2014.09.007.
60. Goymer P, Kahn SG, Malone JG, Gehrig SM, Spiers AJ, Rainey PB. 2006. Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. II. Role of the GGDEF regulator WspR in evolution and development of the wrinkly spreader phenotype. Genetics 173:515–526. https://doi.org/10.1534/genetics.106.055863.
61. Rainey PB, Travisano M. 1998. Adaptive radiation in a heterogeneous environment. Nature 394:69–72. https://doi.org/10.1038/27900.
62. Bantinaki E, Kassen R, Knight CG, Robinson Z, Spiers AJ, Rainey PB. 2007. Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. III. Mutational origins of wrinkly spreader diversity. Genetics 176:441–453. https://doi.org/10.1534/genetics.106.069906.
63. McDonald MJ, Gehrig SM, Meintjes PL, Zhang X-X, Rainey PB. 2009. Adaptive divergence in experimental populations of *Pseudomonas fluorescens*. IV. Genetic constraints guide evolutionary trajectories in a parallel adaptive radiation. Genetics 183:1041–1053. https://doi.org/10.1534/genetics.109.107110.
64. Lind PA, Farr AD, Rainey PB. 2015. Experimental evolution reveals hidden diversity in evolutionary pathways. Elife 4:e07074. https://doi.org/10.7554/eLife.07074.
65. Sánchez-Contreras M, Martín M, Villacieros M, O'Gara F, Bonilla I, Rivilla R. 2002. Phenotypic selection and phase variation occur during alfalfa root colonization by Pseudomonas fluorescens F113. J Bacteriol 184:1587–1596. https://doi.org/10.1128/JB.184.6.1587-1596.2002.
66. Leoni L, Orsi N, de Lorenzo V, Visca P. 2000. Functional analysis of PvdS, an iron starvation sigma factor of *Pseudomonas aeruginosa*. J Bacteriol 182:1481–1491. https://doi.org/10.1128/JB.182.6.1481-1491.2000.
67. Dybvig K. 1993. DNA rearrangements and phenotypic switching in prokaryotes. Mol Microbiol 10:465–471. https://doi.org/10.1111/j.1365-2958.1993.tb00919.x.
68. Irvine S, Bunk B, Bayes HK, Spröer C, Connolly JPR, Six A, Evans TJ, Roe AJ, Overmann J, Walker D. 2019. Genomic and transcriptomic characterization of *Pseudomonas aeruginosa* small colony variants derived from a chronic infection model. Microb Genom 5:e000262. https://doi.org/10.1099/mgen.0.000262.
69. Liberati NT, Urbach JM, Miyata S, Lee DG, Drenkard E, Wu G, Villanueva J, Wei T, Ausubel FM. 2006. An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. Proc Natl Acad Sci U S A 103:2833–2838. https://doi.org/10.1073/pnas.0511100103.
70. Rybtke M, Chua SL, Yam JKH, Givskov M, Yang L, Tolker-Nielsen T. 2017. Gauging and visualizing c-di-GMP levels in *Pseudomonas aeruginosa* using fluorescence-based biosensors. Methods Mol Biol 1657:87–98. https://doi.org/10.1007/978-1-4939-7240-1_8.
71. Rybtke MT, Borlee BR, Murakami K, Irie Y, Hentzer M, Nielsen TE, Givskov M, Parsek MR, Tolker-Nielsen T. 2012. Fluorescence-based reporter for gauging cyclic di-GMP levels in *Pseudomonas aeruginosa*. Appl Environ Microbiol 78:5060–5069. https://doi.org/10.1128/AEM.00414-12.
72. Choi KH, Kumar A, Schweizer HP. 2006. A 10-min method for preparation of highly electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J Microbiol Methods 64:391–397. https://doi.org/10.1016/j.mimet.2005.06.001.
73. RStudio Team. 2020. RStudio: integrated development environment for R, v1.3.1093. http://www.rstudio.com/.
74. Chen S, Zhou Y, Chen Y, Gu J. 2018. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34:i884–i890. https://doi.org/10.1093/bioinformatics/bty560.
75. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069. https://doi.org/10.1093/bioinformatics/btu153.
76. Wolter DJ, Emerson JC, McNamara S, Buccat AM, Qin X, Cochrane E, Houston LS, Rogers GB, Marsh P, Prehar K, Pope CE, Blackledge M, Déziel E, Bruce KD, Ramsey BW, Gibson RL, Burns JL, Hoffman LR. 2013. *Staphylococcus aureus* small-colony variants are independently associated with worse lung disease in children with cystic fibrosis. Clin Infect Dis 57:384–391. https://doi.org/10.1093/cid/cit270.
77. Rahme LG, Stevens EJ, Wolfort SF, Shao J, Tompkins RG, Ausubel FM. 1995. Common virulence factors for bacterial pathogenicity in plants and animals. Science 268:1899–1902. https://doi.org/10.1126/science.7604262.
78. Benie CKD, Dadié A, Guessennd N, N'gbesso-Kouadio NA, Kouame ND, N'golo DC, Aka S, Dako E, Dje KM, Dosso M. 2017. Characterization of virulence potential of *Pseudomonas aeruginosa* isolated from bovine meat, fresh fish, and smoked fish. Eur J Microbiol Immunol 7:55–64. https://doi.org/10.1556/1886.2016.00039.
79. Déziel E, Paquette G, Villemur R, Lepine F, Bisaillon J. 1996. Biosurfactant production by a soil *pseudomonas* strain growing on polycyclic aromatic hydrocarbons. Appl Environ Microbiol 62:1908–1912. https://doi.org/10.1128/aem.62.6.1908-1912.1996.
80. Guerra-Santos L, Käppeli O, Fiechter A. 1984. *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source. Appl Environ Microbiol 48:301–305. https://doi.org/10.1128/aem.48.2.301-305.1984.
81. Lalancette C, Charron D, Laferrière C, Dolcé P, Déziel E, Prévost M, Bédard E. 2017. Hospital drains as reservoirs of *Pseudomonas aeruginosa*: multiple-locus variable-number of tandem repeats analysis genotypes recovered from faucets, sink surfaces and patients. Pathogens 6:36. https://doi.org/10.3390/pathogens6030036.
82. Pemberton JM, Holloway BW. 1972. Chromosome mapping in *Pseudomonas aeruginosa*. Genet Res 19:251–260. https://doi.org/10.1017/s0016672300014518.