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Gene Expression Profiling of *Burkholderia cenocepacia* at the Time of Cepacia Syndrome: Loss of Motility as a Marker of Poor Prognosis?

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Cepacia syndrome (CS) is a fatal septic condition that develops in approximately 20% of cystic fibrosis (CF) patients chronically infected with the *Burkholderia cepacia* complex (Bcc). The most common causative agent is *Burkholderia cenocepacia*, a clinically dominant Bcc species that contains the globally distributed epidemic strain sequence type 32 (ST32). Using microarrays, we compared the transcriptomes of ST32 isolates from the bloodstream at the time of CS with their sputum counterparts recovered 1 to 2 months prior to the development of CS. Global gene expression profiles of blood isolates revealed greater activities of the virulence genes involved in the type III secretion system, the bacterial exopolysaccharide cepacian, and quorum sensing, while reduced expression was demonstrated for flagellar genes. Furthermore, a nonmotile phenotype (as evaluated by a swimming motility assay) was identified in blood isolates from 6 out of 8 patients with CS; this phenotype was traceable to 24 months prior to the onset of CS. Loss of motility was not observed in any of the 89 ST32 isolates recovered over the course of chronic infection from 17 patients without CS. In conclusion, the gene expression of Bcc bacteria disseminated during CS has been elucidated for the first time. This study demonstrated marked differences at the transcriptome level between isogenic ST32 isolates that are attributable to the stage and site of infection. The finding of a nonmotile *B. cenocepacia* isolate may serve as a warning sign for the development of CS in the near future.

Patients with cystic fibrosis (CF) spend a lifetime at risk of contracting bacteria from the *Burkholderia cepacia* complex (Bcc), consisting of a group of 18 genetically closely related bacterial species (1). These microorganisms usually cause chronic respiratory infections in CF patients and result in little chance of treatment success due to their intrinsic resistance to most antimicrobials (2). Moreover, they pose a high risk for the development of a fatal clinical condition termed cepacia syndrome (CS). Because of this unfavorable outcome, Bcc species are considered particularly troublesome CF pathogens associated with not only increased morbidity but also increased mortality (3). Additionally, they spread relatively easily among CF patients and have resulted in several serious Bcc outbreaks in the past. A multicenter outbreak was reported for epidemic lineage ET12 (4), while in a local epidemic identified at the Prague CF Centre in the early 2000s, 30% of the CF population was found to be infected with a single Bcc strain. This strain was designated by multilocus sequence typing (MLST) (5) as sequence type 32 (ST32).

Cepacia syndrome (CS) is a worrying terminal phase of Bcc infection characterized by pulmonary exacerbation, high levels of inflammatory markers, new multifocal lung infiltrates visible by chest X-ray, and a positive blood culture for Bcc bacteria. CS is linked mostly to *Burkholderia cenocepacia*, a species that is clinically dominant among the Bcc members and that includes the infamous epidemic ET12 clone and ST32 strain. Nevertheless, the capacity to enter the bloodstream from the lung of a CF patient is not restricted to *B. cenocepacia*. Indeed, other Bcc species that have been implicated as the causative agents of sepsis are *Burkholderia dolosa* (6) and *Burkholderia multivorans* (7).

Empirical evidence indicates that approximately 20% of Bcc-infected patients die due to CS and that the preceding duration of the infection can vary from weeks (E. Tullis, unpublished data) to decades (8). Despite its clinical relevance, the mechanisms underlying the onset and development of CS remain unknown. We have been unable to pinpoint any apparent trigger or predict for which patients and under which circumstances the greatest risk of CS occurs.

To unravel the bacterial factors that are possibly associated with CS, we performed a microarray-based set of experiments to investigate the global gene expression changes characteristic of *B. cenocepacia* strain ST32. The bloodstream bacterial isolates were found to express higher levels of genes involved in the type III secretion system and the exopolysaccharide cepacian and suppress the activity of the flagellar system. Interestingly, changes in bacterial motility were almost exclusively observed in cases of ST32 infection that resulted in CS.

MATERIALS AND METHODS

Patients and bacterial isolates. This retrospective study included 128 sputum and 9 blood isolates of *B. cenocepacia* ST32 recovered from 25 adult CF patients (8 of them presented with CS with blood culture posi-

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tivity) (Table 1; see also Table S1 in the supplemental material). As part of our routine microbiological investigation of CF samples, each isolate was identified based on its growth on *B. cepacia* selective agar (BCSA; Oxoid) and confirmed by Bcc-specific PCR. Species and strain identifications were determined by MLST or ST32-specific PCR (9).

Global gene expression analyses were performed on two pairs of ST32 isolates from two patients (patient A, a female who died after 16 years of Bcc infection, and patient B, a male who died after 12 years of Bcc infection). One isolate per pair was recovered from blood at the time of CS (isolates A3 and B8 [Table 1]), while the other (isolates A2 and B7) was isolated from sputum 43 and 28 days earlier, respectively.

Microarrays. (i) Experimental design. We worked with two-color custom 4x44K microarrays (ArrayExpress A-MEXP-1613; Agilent Technologies) designed for *B. cenocepacia* strain J2315 (10) and completed with unique features of CF strain AU1054 (11). The experimental layout and raw data were stored in ArrayExpress E-MTAB-3133. Briefly, we ran 36 microarrays, including 9 arrays for each of the 4 isolates tested. A Cy3 layer of the microarrays was occupied with Cy3-labeled J2315 DNA as a reference as described in reference 12 and used for robust signal normalization across all of the arrays.

(ii) CF sputum and serum samples. Each type of clinical specimen used for the production of incubation medium was obtained from adult CF patients who provided informed consent. Sputum samples were immediately transferred to a −20°C freezer; whole-blood samples were left up to 5 days at 4°C. Separated serum was kept at −20°C.

(iii) Growth conditions. Every isolate was subjected in triplicate to the following incubation conditions: (i) 10% (wt/vol) pooled CF sputum diluted in a basal salts medium (BSM) (13) (growth condition "sputum"), (ii) heat-inactivated (by exposure to 56°C for 30 min) pooled CF serum (growth condition "serum"), and (iii) BSM ("control"). For incubation in CF sputum, we followed a previously published protocol (13) with several modifications: (i) pooling of sputa or sera from 5 patients, (ii) total length of incubation set to 270 min, and (iii) processing after the cool down in liquid nitrogen, followed by centrifugation (5 min at 160 × *g*, 4°C), pellet vortexing with 1 ml of TRIzol (Ambion), and incubation at an ambient temperature for 5 min. Next, 0.2 ml of chloroform was added, the samples were incubated for 3 min and centrifuged for 15 min (160 × *g*, 4°C), and the upper water phase was transferred to 500 μl of ice-cooled 70% ethanol.

(iv) RNA processing. RNA was extracted with the RiboPure-Bacteria kit (Ambion), incubated with DNase I (Ambion) for 1 h at 37°C, concentrated with 7.5 M lithium chloride (Ambion), and purified with the MICROBEnrich kit (Ambion) according to the manufacturer's instructions. The quality of the resulting mRNA was determined using total RNA Nano chips on a Bioanalyzer 2100 (Agilent). To label the samples with fluorescent dye, we applied the SuperScript Indirect cDNA labeling system (Invitrogen) and Cy5 or Cy3 dye (GE Healthcare). The hybridization conditions and the washing and scanning of the microarrays were performed as previously described (13).

(v) Analysis. To preprocess the microarray data, we imported the data into *R* (www.R-project.org) using the function read.maimages from the package limma (14). Data were background corrected, normalized within arrays by loess, and normalized between arrays using the Gquantile method. Normalized red-channel data were analyzed with GeneSpring GX v.12 (Agilent) as single-color microarray data. Genes with statistically significant alterations in expression were identified by applying a 2-fold change filter and an adjusted *P* value of 0.05 (moderated *t* test, Benjamini Hochberg false-discovery rate [FDR]).

Swimming motility assay. To measure bacterial swimming activity, we used Luria-Bertani (LB) broth plates containing 0.3% (wt/vol) Bacto agar (Sigma-Aldrich). We inoculated 2 μl of bacteria grown in LB broth (optical density at 260 nm [OD_{600}], 1) into the central part of the soft agar by piercing the agar surface and letting the bacteria grow at 37°C for up to 120 h. The extent of motility was determined by measuring the diameter of the bacterial halos. For the purpose of this study, we defined a consid-

TABLE 1 *B. cenocepacia* ST32 isolates used in this study from patients with CS

Patient with CS	Site[a] and collection date (mo/yr) for sequential isolate no.:								
	1	2	3	4	5	6	7	8	9
A	Sputum 1/2002	Sputum 1/2010	Blood 3/2010						
B	Sputum 3/2003	Sputum 10/2008	Sputum 1/2009	Sputum 10/2009	Sputum 2/2010	Sputum 7/2010	Sputum 10/2010	Blood 11/2010	
C	Sputum 3/2002	Sputum 1/2009	Sputum 11/2009	Sputum 2/2010	Sputum 12/2011	Blood 2/2012			
D	Sputum 1/2002	Sputum 12/2006	Sputum 04/2007	Sputum 1/2009	Sputum 10/2009	Sputum 2/2010	Blood 4/2010	Blood 4/2010	
E	Sputum 1/2004	Sputum 9/2011	Sputum 4/2012	Sputum 4/2013	Sputum 10/2013	Blood 10/2013			
F	Sputum 11/2009	Sputum 2/2011	Blood 3/2011						
G	Sputum 3/2002	Sputum 10/2009	Sputum 2/2010	Sputum 8/2010	Blood 11/2010				
H	Sputum 3/2006	Sputum 5/2006	Sputum 5/2007	Sputum 1/2008	Sputum 10/2009	Sputum 5/2010	Blood 11/2010	Sputum 12/2010	Sputum 4/2011

[a] Isolates were recovered from a sputum sample ("sputum") or blood culture ("blood").

TABLE 2 Summary of microarray data analyses applied and the number of genes with altered expression when focused on growth condition[a]

Parameter	No. of altered genes	No. of upregulated genes	No. of downregulated genes
Growth condition sputum			
Isolates obtained from blood culture			
A3 and B8 isolates together	30	22	8
A3 only	61	37	24
B8 only	163	93	70
Isolates obtained from sputum culture			
A2 and B7 isolates together	208	89	119
A2 only	262	141	121
B7 only	359	153	206
Isolates obtained from both blood and sputum			
A3, B8, A2, and B7	157	70	87
Growth condition serum			
Isolates obtained from blood culture			
A3 and B8 isolates together	169	90	79
A3 only	67	33	34
B8 only	304	137	167
Isolates obtained from sputum culture			
A2 and B7 isolates together	374	177	197
A2 only	488	200	288
B7 only	381	186	195
Isolates obtained from both blood and sputum			
A3, B8, A2, and B7	265	134	131

[a] Gene changes observed while the same isolate was incubated in sputum or serum compared with the control medium.

erable decrease in motility as a first occurrence of nonmotility or decrease in motility by a minimum of 40 mm over the last 12 months.

Quantitative PCR. Changes in gene expression were examined for 6 selected genes in 21 longitudinal isolates collected from 8 patients with CS. The minimal time interval between the collections of two isolates from a single patient was set to 1 month.

All isolates were cultivated in BSM and their RNAs were extracted as described above. cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen), and the PCR was performed using the QuantiTect SYBR green PCR kit (Qiagen). The *recA* gene was used as a reference based on its stable and unchanged expression across the conditions tested by microarrays.

RESULTS AND DISCUSSION

Microarray analysis. To compare transcriptomes, we chose a hybridization-based approach that has been frequently applied in Bcc research (15). Because we utilized a microarray designed for *B. cenocepacia* strain J2315 (a member of ET12), we first evaluated its suitability for the ST32 strain. Based on preliminary results from

TABLE 3 Summary of microarray data analyses applied and the number of genes with altered expression when focused on the site of infection[a]

Parameter	No. of altered genes	No. of upregulated genes	No. of downregulated genes
Incubated in sputum			
Isolates of patients A and B together (A3 and B8 vs A2 and B7)	41	31	10
Isolates of patient A only (A3 vs A2)	210	113	97
Isolates of patient B only (B8 vs B7)	139	90	49
Incubated in serum			
Isolates of patients A and B together (A3 and B8 vs A2 and B7)	20	13	7
Isolates of patient A only (A3 vs A2)	145	99	46
Isolates of patient B only (B8 vs B7)	90	47	53
Incubated in control medium			
Isolates of patients A and B together (A3 and B8 vs A2 and B7)	57	24	33
Isolates of patient A only (A3 vs A2)	34	28	6
Isolates of patient B only (B8 vs B7)	188	92	96
Irrespective of culture medium			
Isolates of patients A and B together (A3 and B8 vs A2 and B7)	60	41	19
Isolates of patient A only (A3 vs A2)	95	65	30
Isolates of patient B only (B8 vs B7)	129	63	66

[a] Gene changes observed between blood and sputum isolates.

TABLE 4 Genes resulting from comparison of blood isolates with sputum isolates[a]

Gene	Annotation or putative gene function	Fold change
Metal ion transport or metabolism		
BCAM2233	Putative pyochelin biosynthetic protein PchC	2.7 ↓
BCAM0810	Putative aromatic oxygenase	2.5 ↑
BCAM0811	Putative aromatic oxygenase	3.1 ↑
Carbohydrate transport and metabolism		
BCAL2793	Major facilitator superfamily protein	3.5 ↓
BCAL2472	Alpha, alpha-trehalose-phosphate synthase, *otsA*	2.1 ↑
BCAM0783	Major facilitator superfamily protein	3.4 ↑
Cell envelope biogenesis, membrane proteins		
BCAM0081	Putative outer membrane protein-OmpW family	2.4 ↓
BCAL1391	Cellulose biosynthesis protein	2.1 ↓
BCAL1395	Cellulose synthase catalytic subunit, *bscA*	2.9 ↓
BCAL1396	Cellulose synthase operon protein YhjU	2.2 ↓
BCAM0805	Muconate cycloisomerase I 1, *catB1*	2.9 ↑
BCAM1004	GDP-mannose 4,6-dehydratase, *gca*	2.6 ↑
BCAM1008	Glycosyltransferase	2.9 ↑
BCAM1010	Putative UTP-glucose-1-phosphate uridylyltransferase, *gtaB*	3.5 ↑
Lipid transport and metabolism		
BCAM0080	AMP-binding domain-containing protein	2.7 ↓
BCAM1005	Putative acyltransferase	3.2 ↑
Energy production		
BCAL2795	Aldehyde dehydrogenase family protein	2.1 ↑
BCAL3184	Homogentisate 1,2-dioxygenase, *hmgA*	3.2 ↑
BCAL3187	FAD[b]-dependent oxidoreductase	2.4 ↑
Regulatory proteins		
BCAL0534	Two-component regulatory system, response regulator protein	2.1 ↓
BCAM2329	GntR family regulatory protein	2.0 ↓
BCAL3205a	Putative phage-related regulator	2.1 ↓
BCAM1290	RpiR-family transcriptional regulator	2.2 ↓
BCAM0835	AraC family regulatory protein	3.7 ↓
BCAM0784	LysR family regulatory protein	2.0 ↑
Quorum sensing		
BCAM1870	*N*-Acyl-homoserine lactone synthase CepI	3.9 ↑
BCAM1871	Hypothetical protein	4.5 ↑
Secretion		
BCAM2041	Yop virulence translocation protein R	4.2 ↑
BCAM2042	Lytic transglycosylase	4.1 ↑
BCAM2043_J_0	Putative uncharacterized protein	2.4 ↑
BCAM2044	Asparagine synthetase	3.0 ↑
BCAM2046	Type III secretion protein SctT	2.6 ↑
BCAM2048	Type III secretion apparatus H^+-transporting two-sector ATPase	4.9 ↑
BCAM2049	BcscL, type III secretion apparatus protein	4.9 ↑
BCAM2050	BcscK, putative uncharacterized protein	7.3 ↑
BCAM2051	Type III secretion apparatus lipoprotein	3.8 ↑
BCAM2052	Uncharacterized protein	3.4 ↑
BCAM2053	Uncharacterized protein	6.9 ↑
BCAM2055	Type III secretion outer membrane pore	4.0 ↑
Exopolysaccharide		
BCAM0859	Capsular exopolysaccharide family	2.8 ↑
BCAM0860	Glycosyl transferase	4.7 ↑
BCAM0861	Glycosyltransferase-like protein	3.1 ↑
BCAM0863	Glycosyltransferase	3.0 ↑

(Continued on following page)

TABLE 4 (Continued)

Gene	Annotation or putative gene function	Fold change
BCAM0864	Glycosyltransferase	2.0 ↑
BCAM0856_J_0	Sugar transferase	2.1 ↑
BCAM0856_J_1	Sugar transferase	4.9 ↑
Motility and adherence		
BCAL1677	Type 1 fimbrial protein	3.4 ↓
Biofilm		
BCAM0184	Fucose-binding lectin II, photopexin A	3.3 ↑
Other proteins		
BCAL2025	Hypothetical protein	2.0 ↓
BCAL2793	Major facilitator superfamily protein	3.5 ↓
BCAL3207	Hypothetical protein	2.5 ↓
BCAM0414	Hypothetical protein	2.3 ↓
BCAM2685	Hypothetical protein	2.6 ↓
BCAL3006	Cold shock-like protein	2.2 ↑
BCAL3186	Hypothetical protein	2.4 ↑

[a] Irrespective of culture medium used, defined in Table 3.
[b] FAD, flavin adenine dinucleotide.

ST32 genome sequencing (provided by M. Kolar and H. Strnad from IMG, unpublished data), the J2315 chip possessed 6,755 probes with close matches to ST32 sequences. Of these, 5,436 represented putative coding sequences (CDS), and 1,049 were localized within intergenic regions. Presuming that the ST32 strain has a total of 7,150 CDS (based on results of *in silico* comparative analysis of the ST32 sequence with the whole genomes of the J2315 [10], HI2424, and AU1054 [11] strains), the microarray used in this study enabled the detection of approximately 76% of all ST32 CDS. This calculation revealed an apparent limitation of the microarray-generated data in that one-quarter of the ST32-specific genes would be absent from the array. However, in this study, we aimed to identify key virulence factors; most of these genes are likely common between the genomes of the highly problematic *B. cenocepacia* clinical strains and, thus, are detectable using this array. Furthermore, analogous data-mining approaches for strains other than J2315 were proven to be valid in several other studies (performed for the H111 [16] and IST439 [17] strains).

A total of 36 arrays were performed to capture expression profiles from two sputum and two blood culture isolates that were each incubated in triplicate in (i) sputum, (ii) serum, and (iii) control medium. The four isolates formed two pairs that had been recovered from two adult CF patients. While the first isolates per pair were obtained from sputum samples (isolates A2 and B7 [Table 1]), the remaining two isolates (isolates A3 and B8) came from blood cultures at the time of CS.

Our experimental layout enabled the comparison of two different test conditions and the identification of changes in gene expression as consequences of either bacterial exposure to laboratory-defined growth conditions (sputum or serum) or the site of infection from which they were recovered (lung or bloodstream). The number of genes with altered expression levels for either of the two conditions is summarized in Tables 2 and 3; these tables also propose various meaningful subvariants of the analyses for each condition (i.e., merged or broken down to individual patients).

With respect to the aim of the study, we focused on the biological origin of the isolate to pinpoint differences in gene activities of the ST32 isolate found in blood (expression changes inherent to CS) as opposed to the ST32 isolate isolated from a CF patient's lung (changes typical of localized infection). Specifically, we performed a subanalysis comparing 18 technical replicates of blood isolates (a combined set of two isolates in growth medium sputum, serum, and control; each in triplicate) with 18 technical replicates of sputum isolates (a subanalysis represented in the "Irrespective of culture medium" rows of Table 3). This approach filtered out any possible effects of the growth media that mimicked *in vivo* conditions and revealed 60 genes with minimal 2-fold changes and *P* values lower than 0.05 in their expression (Table 4).

Changes in expression of virulence factors. Blood isolates were characterized by increased expression of the gene clusters for the type III secretion system (T3SS) and the exopolysaccharide cepacian. They also overexpressed the quorum-sensing (QS) gene *cepI*. Interestingly, the activities of genes for the cepacian and other surface polysaccharides were most noticeable during growth in sputum (Table 2, "Growth condition sputum" rows), while T3SS activities were most pronounced in the serum environment (Table 2, "Growth condition serum" rows). Genes for flagellar assembly and siderophore pyochelin emerged from the list of downregulated genetic features. Indeed, clusters of flagellar genes (see Table S2 in the supplemental material) were found to exhibit significantly decreased expressions only in blood isolate A3 but not in B8 (discussed below).

Confirmatory quantitative PCR for the T3SS and cepacian genes provided results consistent with the microarray data for isolates of all but one patient with CS (see Table S3 in the supplemental material). Variability in the quantitative PCR results for the QS genes and nonuniformity across the isolates likely reflected a role for QS as a global regulator whose expression is very sensitive to conditions outside the cell (15, 18). Nevertheless, the expression of QS genes in isolates from patients A and B matched the microarray data.

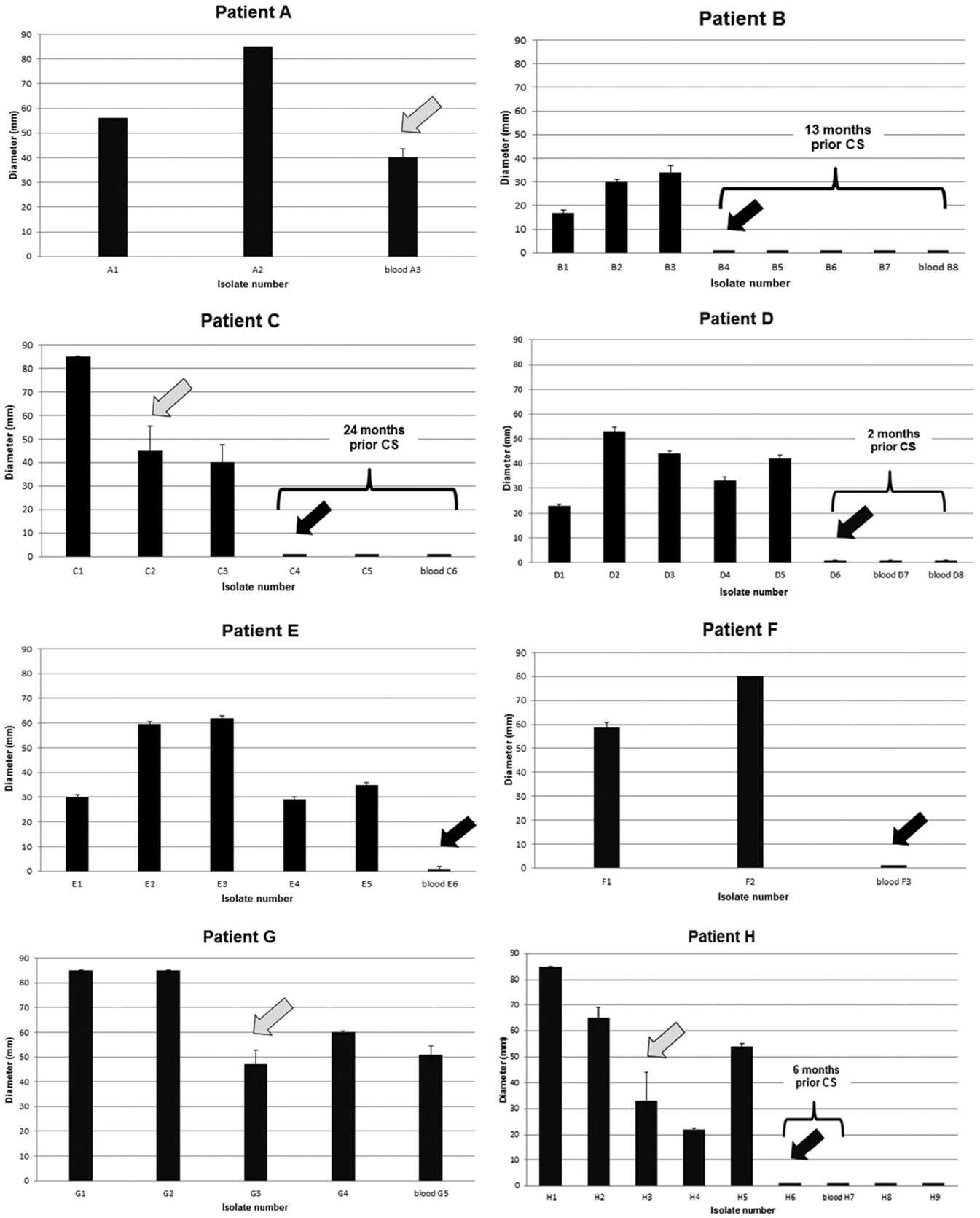

FIG 1 Swimming motility of isolates from patients A through H with CS. Detected changes in motility status due to nonmotility or a substantial decrease in motility are indicated with a black or gray arrow, respectively. The time period preceding CS is shown only for cases where a nonmotile sputum isolate was detected prior to CS.

Virulence factors, such as motility, T3SS, and exopolysaccharides, are QS dependent, and their orchestrated regulation in Bcc bacteria was previously reported (19, 20). In the context of our test condition (the biological origin of the isolate), we presumed that their significant gene expression changes had important implications for survival in the bloodstream and contributed to bacterial cell defense against the host immune system. This notion was supported by studies on another CF respiratory pathogen, *Pseudomonas aeruginosa*, which highlighted the protective role of T3SS (21) and a nonmotile phenotype (22) against phagocytosis. Moreover, mucoidity associated with the production of cepacian was reported to be an important factor for *B. cenocepacia* resistance to phagocytosis (23).

Flagella and their link to CS. Our previous transcriptomic data from a CF sputum-based infection model indicated that the Bcc bacteria maintained their motility during chronic infection, in contrast to *P. aeruginosa* (13). Therefore, the downregulation of the flagellar genes in blood isolate A3 was an unexpected observation, but it was confirmed by a follow-up experiment in which a steep decrease in flagellum-mediated motility between A2 and A3 was verified using a swimming motility assay (Fig. 1).

Because the expression changes for flagellar genes were detectable only by microarray between the two isolates of patient A, we hypothesize that the functions of the flagella in patient B may have already been altered in earlier sputum isolates. Indeed, when we screened isolates preceding the most recent sputum isolate (B7), we discovered a nonmotile phenotype as early as 13 months prior to CS. Subsequently, the loss of motility was identified in longitudinal data from 6 of 8 patients who developed CS; isolates of the 2 remaining patients (A and G) presented a rapid decrease in their motility (>40-mm reductions of the growth zone; Fig. 1). The maximum time interval between the first detected nonmotile isolate and the last blood culture isolate was 24 months. In contrast, isolates from 17 patients who suffered from chronic ST32 infection without signs of CS (see Table S1 in the supplemental material) were all found to be motile (see Fig. S1; infection span, 4 to 12 years). A considerable and maintained decrease in motility was detected in 18% of the patients (patients L, U, and W).

These findings suggest a link between nonmotility (or a rapid change in *B. cenocepacia* ST32 motility) and CS ($P = 0.0002$, Fisher's exact test). Nonetheless, it is noteworthy that such data need to be considered strictly strain specific. A recent study on a large collection of various *B. cenocepacia* CF strains did not find any correlation between motility and clinical outcomes, despite the fact that 38% of the tested isolates were nonmotile (24).

We believe that a simple phenotypic test to investigate the swimming activity of ST32 isolates can serve as a useful tool for monitoring chronic infection and help identify patients at the greatest risk for developing CS, which is currently the case for patients L, U, and W, whose latest isolates expressed a marked decrease in motility (see Fig. S1 in the supplemental material).

In conclusion, we performed a thorough transcriptomic analysis of *B. cenocepacia* isolates recovered at the terminal stage of chronic infection in CF patients. Bacterial isolates from the bloodstream exhibited upregulated gene expressions for T3SS and cepacians compared to isolates from sputum before the onset of CS and showed reduced expressions of flagellar genes; this loss of motility was maintained in sputum isolates up to 24 months prior to the development of CS.

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