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The Genome of *Burkholderia cenocepacia* J2315, an Epidemic Pathogen of Cystic Fibrosis Patients[▽][†]

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Bacterial infections of the lungs of cystic fibrosis (CF) patients cause major complications in the treatment of this common genetic disease. *Burkholderia cenocepacia* infection is particularly problematic since this organism has high levels of antibiotic resistance, making it difficult to eradicate; the resulting chronic infections are associated with severe declines in lung function and increased mortality rates. *B. cenocepacia* strain J2315 was isolated from a CF patient and is a member of the epidemic ET12 lineage that originated in Canada or the United Kingdom and spread to Europe. The 8.06-Mb genome of this highly transmissible pathogen comprises three circular chromosomes and a plasmid and encodes a broad array of functions typical of this metabolically versatile genus, as well as numerous virulence and drug resistance functions. Although *B. cenocepacia* strains can be isolated from soil and can be pathogenic to both plants and man, J2315 is representative of a lineage of *B. cenocepacia* rarely isolated from the environment and which spreads between CF patients. Comparative analysis revealed that ca. 21% of the genome is unique in comparison to other strains of *B. cenocepacia*, highlighting the genomic plasticity of this species. Pseudogenes in virulence determinants suggest that the pathogenic response of J2315 may have been recently selected to promote persistence in the CF lung. The J2315 genome contains evidence that its unique and highly adapted genetic content has played a significant role in its success as an epidemic CF pathogen.

Burkholderia cenocepacia is the most clinically important member of *B. cepacia* complex (BCC) group of opportunistic pathogens to cause lung infections in cystic fibrosis (CF) patients (83, 140). The BCC (originally described as *Pseudomonas cepacia*) emerged as significant CF pathogens in the early 1980s when a minority of infected patients exhibited rapid clinical deterioration due to necrotizing pneumonia and sepsis, resulting in early death (54). This fatal decline in clinical condition became known as "cepacia syndrome" and has not been observed with any other CF pathogen. The key determinants associated with this syndrome are not clear; clonal isolates can be isolated from patients with or without cepacia syndrome, suggesting that both bacterial and host factors play important roles in determining clinical prognosis (44, 57). During the 1990s a highly transmissible epidemic *B. cenocepacia* lineage emerged that was readily spread between individuals with CF (44); multilocus enzyme electrophoresis designated it as electrophoretic type 12 (ET12) (56). Subsequent studies showed that this *B. cenocepacia* strain was widespread across Canadian (80, 126), United Kingdom (44), and European (140) CF communities and was suggested to have spread through patient-to-patient contacts, including those at CF summer camps (44).

At least 17 species comprise the BCC (79, 141, 142), a diverse collection of genetically distinct but phenotypically similar strains that includes bioremediation and biocontrol strains, as well as plant, animal, and human pathogens (83). Although strains from each of the BCC species have been isolated from

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CF infection (83), epidemics are largely attributed to *B. cenocepacia* (25, 27) and *Burkholderia multivorans* (120) strains. Phylogenetic analysis of the *recA* gene subdivides *B. cenocepacia* into four distinct subgroups (141), with subgroup IIIA containing the ET12 epidemic strain, associated with "cepacia syndrome" (54), clinical deterioration, increased mortality (44, 57), and the ability to superinfect over existing *B. multivorans* lung infection (84). Virulence markers, such as the *B. cepacia* epidemic strain marker and the *B. cenocepacia* island (CCI), are more frequently associated with *B. cenocepacia* IIIA strains than other *B. cenocepacia* *recA* subgroups (10). In addition, ET12 isolates possess the cable pilus (83, 137). Unlike other *B. cenocepacia* subgroups, which can frequently be recovered from the natural environment (31, 70), very few environmental *B. cenocepacia* IIIA strains have been described (8), suggesting a definite shift from a soil saprophyte to host-associated pathogen lifestyle.

The genome of *B. cenocepacia* strain J2315, a multidrug-resistant CF patient isolate belonging to the ET12 lineage (44, 100), has been sequenced. The genomic analysis of *B. cenocepacia* J2315 provides insights into the success of this strain and how the ET12 lineage appears to have recently adapted to its clinical niche in human infection.

MATERIALS AND METHODS

Bacterial strains. *B. cenocepacia* strain J2315 (CF5610) was isolated in 1989 from the sputum of a CF patient in Edinburgh, who was the United Kingdom index case of the highly transmissible ET12 lineage (44). J2315 is resistant to the aminoglycosides amikacin and tobramycin, the macrolide azithromycin, the β-lactams imipenem and piperacillin, and cotrimoxazole (trimethoprim-sulfamethoxazole) and also exhibits intermediate resistance to the fluoroquinolone ciprofloxacin. Strain J2315 has been deposited as LMG 16656 in the BCCM/LMG Bacteria Collection.

Additional *B. cenocepacia* strains used in the present study were K56-2*, BC7*, LMG 13307 (BCC0162), CEP0791 (BCC0077), LMG 13320 (BCC0179), FC0504 (BCC0313), LMG 18827* (BCC0016), BCC1261, CEP0826 (BCC0222). (An asterisk [*] indicates strains that are part of the published BCC strains panel [81].) All strains had previously been sequence typed (9).

Genome sequencing. (i) Whole-genome sequencing. Strain J2315 was grown, and DNA was extracted exactly as described previously (82). Sequence data were obtained from 215,165 end sequences (giving approximately 11.9-fold coverage) derived from m13mp18 and pUC18 genomic shotgun libraries (with insert sizes of 1 to 6 kb) using BigDye terminator chemistry on ABI 3700 automated sequencers. A total of 4,300 end sequences from a large insert bacterial artificial chromosome library (with insert sizes of 10 to 20 kb) were used as a scaffold. All identified repeats were bridged by read-pairs or end-sequenced PCR products.

The sequence was annotated by using Artemis software (112). Initial coding sequence (CDS) predictions were performed by using Orpheus (40), Glimmer2 (34), and EasyGene (69) software. These predictions were amalgamated, and codon usage, positional base preference methods, and comparisons to the non-redundant protein databases using BLAST (4) and FASTA (106) software were used to refine the predictions. The entire DNA sequence was also compared in all six reading frames against UniProt, using BLASTX (4) to identify any possible CDSs previously missed. Protein motifs were identified by using Pfam (12) and Prosite (37), transmembrane domains were identified with TMHMM (65), and signal sequences were identified with SignalP version 2.0 (98). rRNAs were identified by using BLASTN (4) alignment to defined rRNAs from the EMBL nucleotide database; tRNAs were identified by using tRNAscan-SE (75); stable RNAs were identified by using Rfam (46).

(ii) Comparative genomics. Comparison of genome sequences was facilitated by using Artemis Comparison Tool (22), which enabled visualization of BLASTN and TBLASTX comparisons (4). Orthologous proteins were identified as reciprocal best matches by using FASTA (106) with manual curation. Pseudogenes had one or more mutations that would prevent correct translation; all inactivating mutations were checked against original sequencing data.

The J2315 genome was compared to *B. vietnamensis* strain G4 (accession numbers CP000614, CP000615, and CP000616) (97), *B. contaminans* strain 383

(CP000151, CP000152, and CP000153) (127, 141), *B. ambifaria* strain AMMD (CP000440, CP000441, and CP000442) (26), *B. cenocepacia* strains AU1054 (CP000378, CP000379, and CP000380) (http://genome.jgi-psf.org/finished _microbes/burca/burca.home.html) and HI2424 (CP000458, CP000459, and CP000460) (70), *B. pseudomallei* strain K96243 (BX571965 and BX571966) (51), *B. mallei* strain ATCC 23344 (CP000010 and CP000011) (99), *B. thailandensis* strain E264 (CP000086 and CP000085) (148), *B. xenovorans* strain LB400 (CP000270, CP000271, and CP000272) (23), and *Ralstonia solanacearum* strain GMI1000 (118).

PCR screening. PCR amplification was performed by using Platinum *Pfx* DNA polymerase (Invitrogen) according to the supplied protocols, with the optional addition of 1/10 enhancer solution. Amplification consisted of 94°C for 10 min, followed by 40 cycles of 94°C for 30 s, a suitable annealing temperature for 30 s, and 68°C for 1 min per kb. A final extension of 10 min at 68°C was used. The primers, along with the annealing temperature(s) used, were as follows: BCAL3125 (AATCGGAACAGGTTGCACTC and AAACTGGAATGCGAA GATGC), 60°C; BCAL3223 (ACCGATGTCTTCCTGTTTGG and AGCGGA TGGTTCTTGATGAC), 60 to 68°C; BCAL3517 (GCACGTTGATTGTTTCT TTGC and AATCGGGATCGACCTTGAC), 63 to 68°C; BCAM0856 (TCGA AATACTTGTCGCGTTG and ACAGGAAGTGGTAGCCGATG), 68°C; and BCAM2228 (GAACCTGACGGTGCTGAAC and GTAGACGGACAGGTCG AAGC), 68°C.

RESULTS

General features of the J2315 genome. The complete genome of *B. cenocepacia* strain J2315 consists of three circular chromosomes of 3,870,082, 3,217,062, and 875,977 bp and a plasmid of 92,661 bp (Fig. 1). These four replicons encode 3,537, 2,849, 776, and 99 predicted CDSs, respectively (for a summary of the features of the replicons, see Table 1), of which 126 are pseudogenes or partial genes. Identification of essential genes on chromosomes 2 and 3 led to designation of these components of the genome as chromosomes rather than megaplasmids.

Inter-replicon comparisons revealed very little extended similarity except in the regions of the rRNA clusters (data not shown). Intrareplicon comparison revealed that chromosome 1 contains a 57-kb perfect duplication (BCAL0969 to BCAL1026 and BCAL2901 to BCAL2846). The duplicated regions are at different locations on the chromosome, and each contains 57 CDSs, leading to an increase in the gene dosage of CDSs that encode a diverse collection of functions, including molybdopterin biosynthesis proteins, RNase E, ribosomal protein, fatty acid/phospholipid synthesis proteins, sigma-E factor and regulon, GTP-binding protein LepA, signal peptidase I, RNase III, DNA repair protein RecO, elongation factor P, and CDP-diacylglycerol–glycerol-3-phosphate 3-phosphatidyltransferase.

Analysis of the predicted functions of the CDSs on the chromosomes revealed distinct partitioning of functions, as has been previously seen in other *Burkholderiaceae* (51): chromosome 1 contains a higher proportion of CDSs involved in core functions (cell division, central metabolism, and other "housekeeping" functions), whereas chromosomes 2 and 3 contain a greater proportion of CDSs encoding accessory functions, such as protective responses and horizontal gene transfer, and a greater proportion of CDSs with unknown functions (Fig. 2).

Comparative genomics. The J2315 genome was compared to five other complete BCC genomes in the public databases: *Burkholderia vietnamiensis* strain G4, an aromatic hydrocarbon degrading environmental isolate (97); *Burkholderia contami-*
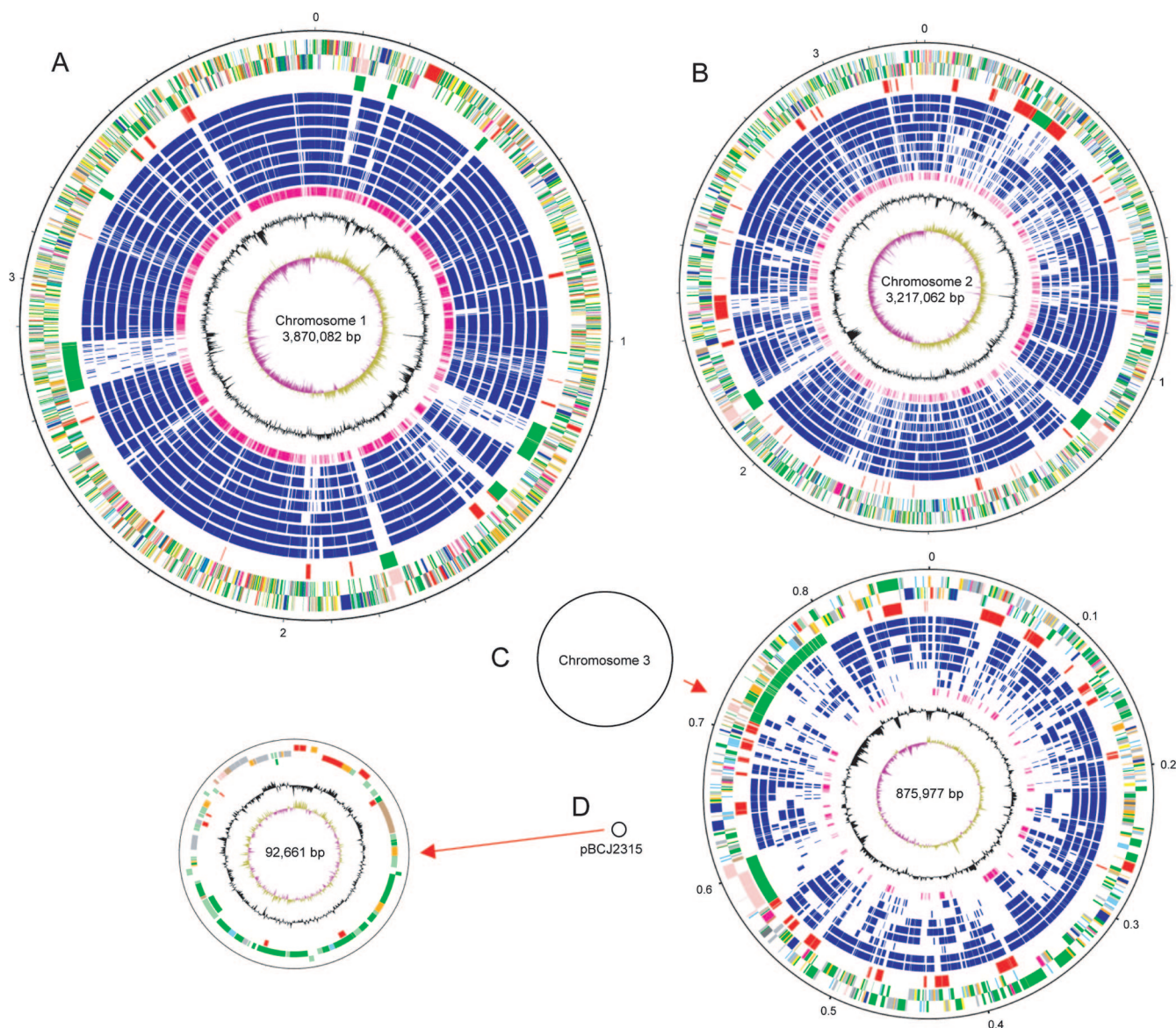
FIG. 1. Schematic circular diagrams of the *B. cenocepacia* J2315 genome. The circular diagrams for chromosomes 1 (A) and 2 (B) are drawn to scale, whereas those for chromosome 3 (C) and plasmid pBCJ2315 (D) are not drawn to scale. Black circles representing these replicons are drawn to scale. The key for the three chromosomal circular diagrams (A, B, and C; outside to inside), with scale in Mb, is as follows. Annotated CDSs are colored according to the predicted function represented on a pair of concentric circles, representing both coding strands. CDSs in genomic island regions are indicated in green, and other RODs defined by pairwise genome comparisons with other BCC are indicated in red. CDSs with matches identified by reciprocal FASTA with other *Burkholderia* species—*B. cenocepacia* HI2424, *B. cenocepacia* AU1054, *B. contaminans* 383, *B. ambifaria* AMMD, *B. vietnamiensis* G4, *B. xenovorans* LB400, *B. pseudomallei* K96243, and *B. thailandensis* E264—are indicated in dark blue. Orthologues shared with *Ralstonia solanacearum* GMI1000 are indicated in purple. For the G+C content plot, the GC bias (G−C/G+C) is indicated as >1% in khaki and <1% in purple. (D) The key for the circular diagram for plasmid pBCJ2315 is as described for the chromosomes but lacks the orthologue matches. Color coding for CDS functions: dark blue for pathogenicity/adaptation, black for energy metabolism, red for information transfer, dark green for surface associated, cyan for degradation of large molecules, magenta for degradation of small molecules, yellow for central/intermediary metabolism, pale green for unknown, pale blue for regulators, orange for conserved hypothetical, brown for pseudogenes, pink for phage and IS elements, and gray for miscellaneous.

nans strain 383, a soil isolate (127, 141); *Burkholderia ambifaria* strain AMMD, a plant-associated biocontrol strain (26); and *B. cenocepacia* IIIB strains AU1054 (http://genome.jgi-psf.org /finished_microbes/burca/burca.home.html) and HI2424 (70), isolated from a CF patient and soil, respectively. Both of these *B. cenocepacia* strains are representatives of the *B. cenocepacia* PHDC clonal lineage (25) that is widely distributed in the

United States (71). In addition, the genomes of four other *Burkholderia* species were compared: *Burkholderia pseudomallei* (51) and *Burkholderia mallei* (99), biothreat agents that cause melioidosis and glanders, respectively; *Burkholderia thailandensis* (148), a soil saprophyte related to *B. pseudomallei* and *B. mallei*; and *Burkholderia xenovorans* (23), a nonpathogenic soil isolate that degrades polychlorinated biphenyl (PCB)

TABLE 1. General properties of the *B. cenocepacia* J2315 genome

Property[a]	Chromosome 1	Chromosome 2	Chromosome 3	Plasmid	Total
Size (bp)	3,870,082	3,217,062	875,977	92,661	8,055,782
G+C content (%)	66.7	67.9	67.9	62.6	66.9
No. of CDSs	3,537	2,849	776	99	7,261
Coding (%)*	86.1	86.4	86.0	78.6	85.9
Avg gene length (bp)*	958	985	986	763	970
rRNA	4 (16S-23S-5S)	1 (16S-23S-5S)	1 (16S-23S-5S)	0	6 (16S-23S-5S)
tRNA	66	6	2	0	74
Miscellaneous RNA	15	3	2	1	21
Pseudogenes and partial genes	56	41	23	6	126
IS elements	51	16	11	1	79

[a] *, Values exclude pseudogenes and partial genes.

compounds. *Ralstonia solanacearum* (118), a plant pathogenic member of the *Burkholderiaceae*, was also included as an outlier.

The number of orthologous CDSs identified by best reciprocal FASTA in the genome of J2315 correlated with taxonomic relatedness (Fig. 3) (141); the largest number of orthologs were identified in the BCC (78 to 63% of total CDSs), followed by other *Burkholderia* species (56 to 50%), and *Ralstonia solanacearum* (37%). The distribution of orthologs on the different replicons is similar to that seen in other *Burkholderia* species, where the level of orthology is greatest on the largest chromosome, with the secondary chromosomes being progressively more divergent (51). The relative diversity of the chromosomes was also evident in pairwise alignments that illustrate regions of similarity and the overall genome structure. A comparison of concatenated genomes of *B. cenocepacia* J2315, *B. pseudomallei* K96243, and *B. xenovorans* LB400 shows that, of all the chromosomes, chromosome 1 displays the greatest level of conservation, both in the number and the order of matches (see Fig. S1 in the supplemental material). The largest chromosomes of these three *Burkholderia* species contain colinear and inverted blocks of similarity, suggesting that these replicons have undergone several rearrangement events since they diverged from a common ancestor. The second chromosome exhibits lower levels of overall conservation. Although there are matches between the third chromosomes of *B. cenocepacia* and *B. xenovorans* (the genome of *B. pseudomallei* only contains two replicons), there is no detectable conservation of gene order.

RODs in the *B. cenocepacia* J2315 genome. Pairwise genome comparisons of the *B. cenocepacia* strain J2315 genome to the other *B. cenocepacia* strains identified regions of difference (RODs) comprising ca. 21% of the DNA in J2315. These include genomic islands (Table 2 and Fig. 1) that are likely to have arisen from recent horizontal gene transfer (9.3% of the chromosomal DNA) and encompasses mobile genetic elements (MGEs). In addition to the genomic islands, the J2315 genome contains 79 insertion sequence (IS) elements (Table 1 and see Table S1 in the supplemental material). Genomic islands were defined as regions displaying anomalies in %G+C content or dinucleotide frequency signature (which is indicative of very recent lateral transfer) and/or contained CDSs with
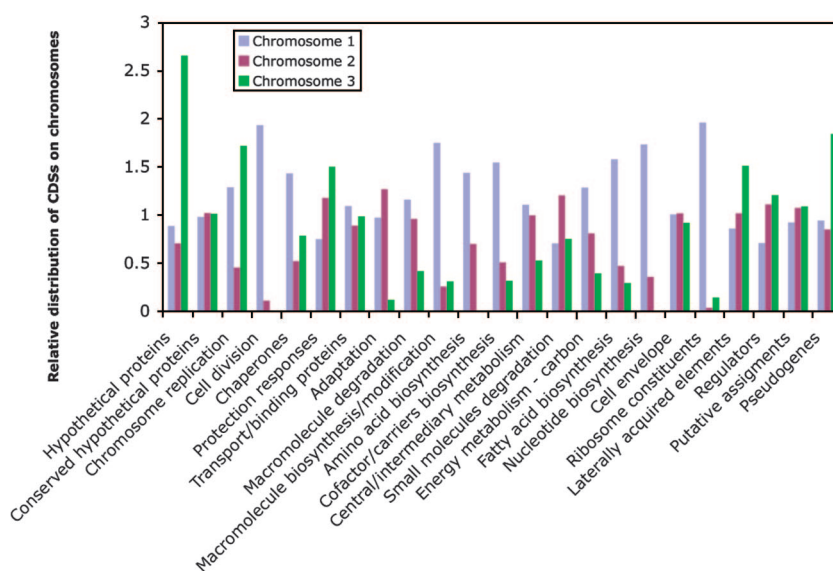

FIG. 2. Relative distribution of CDSs belonging to different functional classes on the three chromosomes of *B. cenocepacia* J2315. Figures for the distribution of the functional classes on a chromosome are expressed as a ratio of the number of CDSs of a class per replicon against the total number CDSs for that class in the genome, normalized per number of CDS on that replicon.
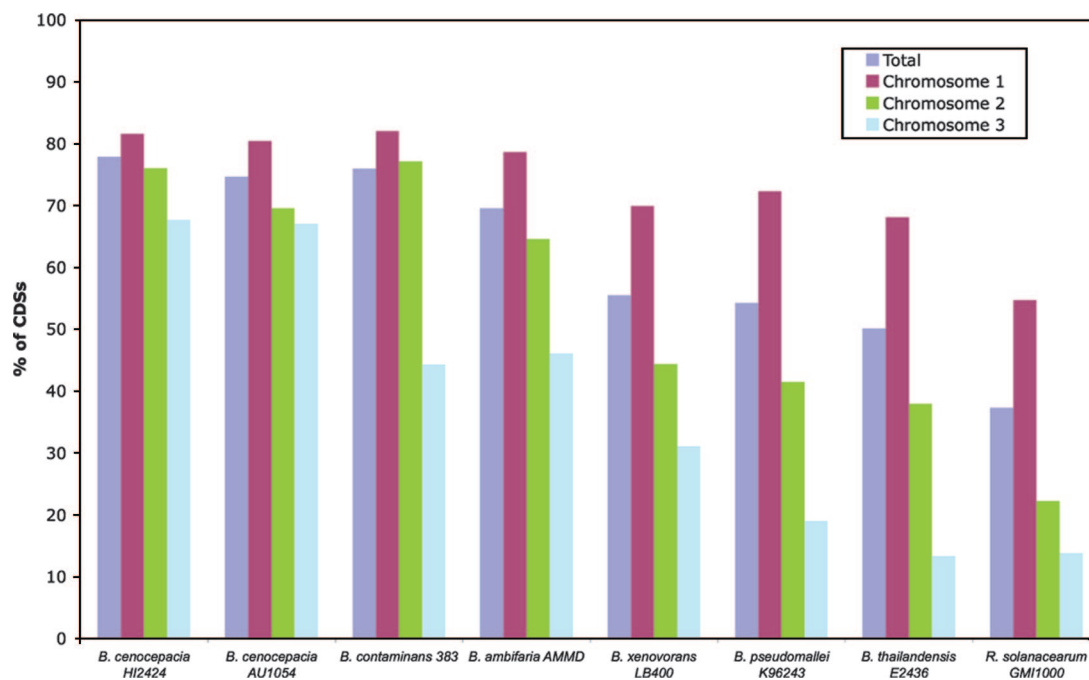
FIG. 3. Comparison of the distribution of *B. cenocepacia* J2315 orthologs. Orthologs were identified in the *Burkholderiaceae* genomes *B. cenocepacia* HI2424, *B. cenocepacia* AU1054, *B. contaminans* 383, *B. ambifaria* AMMD, *B. vietnamiensis* G4, *B. xenovorans* LB400, *B. pseudomallei* K96243, and *B. thailandensis* E264 and in *Ralstonia solanacearum* GMI1000. Orthologs were identified by best reciprocal FASTA with an identity cutoff of 30% and a length of match cutoff of 80%. The percentages of matches for individual J2315 chromosomes and the total genome are plotted.

similarities to genes associated with MGEs such as bacteriophages, transposons, and plasmids. Boundaries of genomic islands were mapped by using comparative genomic analysis. Other RODs in the J2315 genome (11.7% of the chromosomal DNA) include indel regions that represent lineage-specific DNA insertions or deletions and allelic variants that have divergent sequences at the same locus (Fig. 1). For the purpose of our analysis, we have not included RODs that do not include at least one complete CDS. Although these other RODs were identified as being unique in comparison to *B. cenocepacia* strains AU1054 and HI2424, many of these regions are ancestral regions that have been deleted in the other *B. cenocepacia* genomes relative to J2315 and the other BCC. For example, if *B. contaminans* strain 383 is included in the comparison, the unique component of the J2315 genome falls to 7.0% (see Table S2 in the supplemental material).

The ET12 lineage of *B. cenocepacia* emerged recently and proved very successful at spreading between patients and causing disease (83). A possible contributory factor in its rise could be the horizontal transfer of DNA. Fourteen regions in the J2315 genome were identified as putative genomic islands (Table 2), all fourteen of which are absent from the genomes of *B. cenocepacia* AU1054 and HI2424.

The CCI, identified in the J2315 genome as genomic island 11 (BcenGI11; Table 2), has been shown to play a role in infection (10, 84). When originally described, comparative analysis of other BCC genomes was not available to allow the boundaries of the island to be accurately predicted. The current analysis defines the CCI as a 44-kb region, 12 kb and six CDSs larger than its original description (10). New functions attributed to the island include arsenic resistance, antibiotic

resistance, ion and sulfate family transporter, and stress response, in addition to the fatty acid metabolism, amino acid transport and utilization, and various regulators that include an *N*-acyl-homoserine lactone-dependent quorum-sensing system originally described (10).

The genome also provides evidence that the pan genome of *B. cenocepacia* encompasses elements that may circulate in the wider bacterial population, as several of the genomic islands are similar to elements identified in other *Burkholderia* species. The J2315 genome contains at least five prophages, one of which, BcenGI1, exhibits extended mosaic similarity to the φK96243 prophage in the *B. pseudomallei* K96243 genome (BP_GI1; see Fig. S2A in the supplemental material) (51).

Comparative genomic analysis identified related genomic islands that integrate at orthologous sites in different species. BcenGI2 is a 16.4-kb genomic island that contains CDSs with similarity to plasmid conjugal transfer proteins, suggesting that it may be an integrated conjugative element (21). BcenGI2 is integrated at a tRNA[Ala] gene and has some similarity to an island (BP_GI11) integrated at the orthologous site in the *B. pseudomallei* K96243 genome (see Fig. S2B in the supplemental material). This locus may be a hot spot for the traffic of related islands in *Burkholderia*.

Genomic inventory of a versatile pathogen. The recent ecology of the ET12 lineage of *B. cenocepacia* is that of a human-associated pathogen, and as such the genome of strain J2315 appears to be well equipped with functions associated with virulence in the CF lung (for a summary, see Table 3). There is also evidence in the genome of the wider host associations of this species, underlying its environmental origins. Orthologous matches to putative J2315 virulence factors were found in the

TABLE 2. Genomic islands of *B. cenocepacia* strain J2315[a]

Chromosome and island	Size (kb)	Coordinates	Integration site	No. of integrases	%GC	D	K	Functional note(s)
Chromosome 1								
BcenGI1	25.0	100361...125265	tRNA Arg	1	– (68.5)	–	–	Prophage, contains a group II intron; similar to φK96243 from *B. pseudomallei* K96243
BcenGI2	16.4	188011...204429	tRNA Ala	1	+ (55.7)	–	+	ICE element similar to GI11 in *B. pseudomallei* K96243
BcenGI3	13.1	449978...463082			+ (56.5)	+	+	Miscellaneous island, possible remnant, contains type I restriction modification system (BCAL0414, BCAL0418, and BCAL0420)
BcenGI4	4.2	1032360...1036537	tRNA Met	1	+ (42.8)	–	+	Miscellaneous island, contains three CDSs of possible plasmid origin; similar island in *B. vietnamiensis* G4
BcenGI5	92.8	1222566...1315385		1	+ (60.3)	–	+	Miscellaneous island, contains miscellaneous metabolism, including glycosyltransferase (BCAL1147), polysaccharide deacetylase (BCAL1148), putative O-antigen acetylase (BCAL1191), possible hydroxybenzoate degradation components (BCAL1151 to BCAL1162), fusaric acid resistance-family transporter (BCAL1176 to BCAL1178), and glycerate kinase (BCAL1181)
BcenGI6	34.2	1402882...1437088	tRNA Arg	1	+ (58.8)	–	+	Miscellaneous island
BcenGI7	37.7	1728919...1766584	BCAL1558		+ (62.9)	–	+	Prophage, Mu-like
BcenGI8	121.9	2738065...2859923	BCAL2601	1	+ (61.4)	–	+	Miscellaneous island, contains miscellaneous metabolism including D-lactate dehydrogenase (BCAL2487) and putative O-antigen acetylase (BCAL2519), glutathione *S*-transferase (BCAL2539), 3-isopropylmalate dehydratase (BCAL2542 and BCAL2543), MFS transporter (BCAL2525 and BCAL2545), ABC transporter (BCAL2573 to BCAL2576), and nitrilase (BCAL2585)
BcenGI9	16.3	3241588...3257909	tmRNA	1	+ (62.8)	–	–	Miscellaneous island, phage origins, possible phage remnant
BcenGI10	6.3	3368693...3375006	tRNA Gly	1	+ (59.4)	–	+	Miscellaneous island, inserted into divergent region
Chromosome 2								
BcenGI11	44.1	290291...334378			+ (62.0)	–	+	Cenocepacia island, contains arsenic resistance (BCAM0233 to BCAM0235), antibiotic resistance (BCAM0237A), ion and sulfate family transporter (BCAM0238 and BCAM0281), and stress response CDSs (BCAM0276 and BCAM0278)
BcenGI12	46.8	1140183...1186975	tRNA SeC	1	+ (63.2)	–	–	Prophage
BcenGI13	46.2	2091701...2137922	BCAM1874	1	+ (54.3)	–	+	Prophage
Chromosome 3								
BcenGI14	36.7	573207...609954		1	+ (62.8)	–	–	Prophage BcepMu

[a] The number of putative integrases contained within islands is given. The presence (+) or absence (–) of anomalies in the properties of DNA within islands is indicated in the columns marked %GC, D, and K, which represent the G+C content, G+C deviation, and the Karlin dinucleotide frequency signature, respectively. The actual %G+C for an island is indicated in parentheses in the %GC column.

other *Burkholderia* genomes investigated, which included environmental bacteria. For example, ~80% of the J2315 virulence functions have orthologous matches to other *B. cenocepacia* strains, ~74% have matches to *B. contaminans* strain 383, and ~68% have matches to *B. pseudomallei*. Many of these functions therefore represent *Burkholderia*-wide functions, which may promote survival in challenging and complex environments such as the soil and rhizosphere but may also have utility in the CF lung. In addition, comparative analysis has highlighted virulence determinants in variable regions of

the J2315 genome (Table 3 and see Table S2 in the supplemental material), suggesting that this strain may have supplemented its core virulence determinants with accessory virulence functions to enhance its disease causing ability.

Exoenzymes. Exoenzymes produced by *B. cenocepacia* play an important role in modulating host cell interactions. Two secreted zinc metalloproteases, ZmpA and ZmpB (Table 3), have been found to have proteolytic activity against a range of host molecules and have been implicated in the virulence of *B. cenocepacia* (29, 62, 63). Phospholipases are widely distributed

TABLE 3. Potential virulence functions encoded in the genome of *B. cenocepacia* strain J2315

Function	Notes and examples	CDSs[a]
Exoproteins	Zinc metalloproteases ZmpA and ZmpB	BCAS0409 and BCAM2307
	Phospholipases C	BCAL0443; BCAL1046; BCAM0408*; BCAM1969; BCAM2429; BCAM2720
Secretion	Type I secretion (T1S)	BCAM2140 to BCAM2142; BCAM1317 to BCAM1320
	Type II secretion (T2S)	BCAL3447 and BCAL3515 to BCAL3527
	Type III secretion (T3S)	BCAM2045 to BCAM2057
	Type IV secretion (T4S)	BCAM0324 to BCAM0340; pBCA020 to pBCA059
	Type V secretion (T5S)	BCAL3353; BCAM0183; BCAM2169; BCAS0321
	Type VI secretion (T6S)	BCAL0337 to BCAL0351
LPS and capsule	LPS biosynthesis clusters: core, O-antigen and lipid A modification	BCAL2402 to BCAL2408; BCAL3110 to BCAL3125; BCAL1929 to BCAL1935
	Cepacian polysaccharide synthesis and export cluster	BCAM0854 to BCAM0865
	Other potential surface polysaccharides biosynthetic clusters	BCAL3217* to BCAL3246*; BCAM0203 to BCAM0214; BCAM1003 to BCAM1011; BCAM1224 to BCAM1228; BCAM1330* to BCAM1340*; BCAM2032* to BCAM2035*; BCAS0294 to BCAS0297
Adhesins	22-kDa adhesion protein AdhA	BCAM2143*
	BuHA family proteins	BCAM0219*; BCAM0223*, BCAM0224*; BCAM1115*; BCAM2418; BCAS0236; BCAS0335; pBCA011*
Fimbriae and pili	Cable pilus	BCAM2756* to BCAM2762*
	Chaperone-usher type fimbriae	BCAL1677 to BCAL1680; BCAL1826 to BCAL1828; BCAL2634a* to BCAL2637*
	Flp-type pili	BCAL1520 to BCAL1537; BCAS0298 to BCAS0312
	Type IVa pilus	BCAL0959, BCAL3445 to BCAL3447, BCAL0276 to BCAL0278
Quorum sensing	*N*-Acylhomoserine lactone regulons CepIR and CciIR	BCAM1870 and BCAM1868; BCAM0239a* and BCAM0240*
	N-Acylhomoserine lactone dependent regulator	BCAM0188
Siderophores	Ornibactin	BCAL1688 to BCAL1702
	Salicylic acid and pyochelin	BCAM2224 to BCAM2235
Intracellular stress	Natural resistance-associated macrophage proteins	BCAM0836, BCAM1764, BCAM2060, BCAS0630 and BCAS0634*
	Superoxide detoxification: superoxide dismutases, peroxidase/catalase, catalases, thiol peroxidase, alkyl hydroperoxidase	BCAL2643 and BCAL2757; BCAL3299 and BCAM2107*; BCAS0635 and BCAM0931; BCAL3424; BCAM1216 and BCAM1217
Motility	Nitric oxide detoxification, flavohaemoglobin	BCAL3285
	Five gene clusters on chromosome 1 encode the components of a single flagellar system	BCAL0113 to BCAL0114; BCAL0140 to BCAL0144; BCAL0520 to BCAL0527; BCAL0561 to BCAL0577; BCAL3501 to BCAL3507
	Two additional flagellar components on chromosome 2 and chromosome 3	BCAM0987; BCAS0104

[a] An asterisk (*) indicates a gene or genes associated with that function that is absent from both *B. cenocepacia* AU1054 and HI2424 genomes.

in bacteria and have been shown to mediate various cellular functions, including membrane maintenance, cellular turnover, and inflammatory response. Production of phospholipase C is linked with CF isolates from patients of poor clinical status (68). The J2315 genome encodes five homologs of *Pseudomonas aeruginosa* phospholipase C proteins (Table 3). The redundancy of these phospholipid-degrading enzymes suggests that there is some functional specificity. To this end, studies of the Plc-1 and Plc-2 phospholipases C from *B. pseudomallei* (orthologues of which are present in the J2315 genome; BCAL1046 and BCAM2429, respectively) have demonstrated that although they both hydrolyze phospholipid phosphatidylcholine and sphingomyelin, they exhibit marked differences in their cytotoxicity (64).

In addition to the five phospholipase C homologs the J2315 genome contains a putative phosphatidylinositol-specific phospholipase C (PI-PLC; BCAM1969). Analysis of the taxonomic distribution of proteins containing the PI-PLC domain PF04185 reveals a very limited occurrence; among the gram-negative bacteria, *Burkholderia* is the only genus within which these proteins have been found, and among the gram-positive bacteria, they have been found in an actinobacterium and some firmicutes, including pathogenic bacilli, *Staphylococcus aureus*, and *Listeria monocytogenes* (for the species distribution, see http://pfam.sanger.ac.uk/family?acc=PF00388). In this latter group of pathogens, PI-PLCs have been shown to have a role in virulence (146, 149).

The J2315 genome also contains putative secreted proteins similar to virulence factors produced by bacterial plant pathogens associated with the degradation of plant tissue. Two CDSs encode polygalacturonases, exoproteins that degrade pectin, a major component of plant cell walls; BCAM2783 and BCAS0196 are 69.1 and 31.9% identical to polygalacturonases from *R. solanacearum* (43) and *Agrobacterium vitis* (50), respectively. In addition to these degradative enzymes, the genome also contains a locus (BCAM0153 to BCAM0156) encoding additional pectin degradation components.

Secretion. Further evidence of the plant associations of *B. cenocepacia* are found in the secretion systems. The J2315 genome encodes a type IV secretion system (T4SS) associated with disease in plants; the plasmid-encoded T4SS (Table 3) secretes plant cytotoxic proteins responsible for plant tissue watersoaking (*ptw*) on onions (35). In addition, there is a cluster on chromosome 2 (Table 3) that is similar to the *ptw* cluster and other T4SSs. The function of this cluster (*vir*) is unclear: mutants do not affect expression of the *ptw* phenotype. The organization of the *vir* cluster is similar to that of the *virB* clusters of *Brucella abortus* and *Agrobacterium tumefaciens*, although it lacks homologues of *virB5* and *virB7*. The cluster contains two additional CDSs between the *virB6* (BCAM0328) and *virB8* (BCAM0331) homologues, similar to *traF* (BCAM0329) and *traI* (BCAM0330) components of the pSB102 plasmid conjugation system. These *tra* system components are functionally equivalent to *virB5* and *virB7*. It is unlikely that this cluster is functional in J2315, since the *virD4* homologue (BCAM0335) contains a frameshift mutation.

The type III secretion system (T3SS; Table 3) is associated with pathogenesis: mutants in the T3SS demonstrate reduced virulence in a murine model of infection (132) and in a *Caenorhabditis elegans* killing assay (86). T3SSs have been shown to be important in the intracellular survival of several pathogens; however, there is no evidence that this is the case in *B. cenocepacia*: internalization and survival of J2315 T3SS mutants in macrophages was the same as for the wild type (67).

There are four type V secretion (T5S) proteins in the genome (Table 3). Two of these autotransporters contain pertactin domains (Pfam PF03212; BCAL3353 and BCAM0183), and the other two contain hemagglutinin repeat domains (Pfam PF05594; BCAM2169 and BCAS0321).

The extensive array of secretion systems has further been enhanced by the identification of a type VI secretion (T6S) system on chromosome 2 (Table 3). Three T6S system clusters have been identified in *P. aeruginosa* (33), one of which (HSI-I) has been demonstrated to be essential in the chronic rat lung infection model (108). HSI-I exports Hcp1, a hexameric protein that has been detected in pulmonary secretions of CF patients, and Hcp1-specific antibodies detected their in sera (93).

In contrast to the other pathogenic *Burkholderia* sequenced, *B. cenocepacia* J2315 does not exhibit a large redundancy of secretion systems. For example, *B. mallei*, and *B. pseudomallei* possess four and six T6SSs, respectively (119), and two and three T3SSs, respectively (51, 99).

LPS and exopolysaccharide (EPS). Lipopolysaccharide (LPS) produced by *B. cenocepacia* has an important role in both disease and resistance to antimicrobial peptides. The LPS of the ET12 strain C1359 has been demonstrated to be endotoxic and to stimulate tumor necrosis factor production in greater quantities than that of *P. aeruginosa* (121). Three clusters in the J2315 genome are associated with the production of the core (BCAL2402 to BCAL2408), O antigen (BCAL3110 to BCAL3125) (101), and lipid A modification (BCAL1929 to BCAL1935) of LPS. However, strain J2315 has lost its ability to make complete LPS O antigen, due to an IS insertion in the glycosyltransferase BCAL3125 (101).

The structure and composition of *B. cenocepacia* LPS contributes to the intrinsic resistance to aminoglycoside (30, 91)

and polymyxin. The presence of 4-amino-4-deoxy-L-arabinose moieties within the inner core region has been shown to reduce the binding of cationic antibiotics such as polymyxin B to *B. cenocepacia* LPS (74, 122). A locus on chromosome 1 (BCAL1929 to BCAL1935) is similar to a cluster of six CDSs in *E. coli* and *Salmonella* that have been shown to direct the transfer of 4-amino-4-deoxy-L-arabinose to lipid A in polymyxin-resistant mutants (134). Interestingly, in pathogens such as *Salmonella*, polymyxin B acts as an antagonist of LPS pathological activity; however, in *B. cenocepacia* this antibiotic enhances its activity (122). A recent study showed that this cluster is essential for the viability of *B. cenocepacia* and that a reduction in viability was accompanied by changes in cell morphology (102).

A cluster associated with the production of cepacian (*B. cenocepacia*-specific EPS) has been identified (Table 3) (92). J2135 does not produce cepacian; a CDS (BCAM0856) in the cluster contains a frameshift mutation (11-bp deletion). Although J2315 is described phenotypically as nonmucoid (49), the genome contains several other loci that encode putative EPS-related functions (Table 3), suggesting that J2315 may have the ability to produce capsular material under some environmental conditions. One of these clusters (BCAL3217 to BCAL3246) (105) is similar to the capsular polysaccharide cluster of *B. pseudomallei* K96243 (51), containing two regions of similarity (BCAL3217 to BCAL3223 and BCAL3240 to BCAL3246) separated by a block of divergent sequence (BCAL3227 to BCAL3239). This cluster is probably not expressed in J2315 since it contains an IS element that disrupts a putative capsule polysaccharide biosynthesis/export protein (BCAL3223).

Pili, fimbriae, and adhesins. The adherence of pathogenic bacteria to host cells is often associated with pili, fimbriae, and adhesins. These surface-expressed structures can modulate interactions with host cells and other bacterial cells and can target cells to a site of infection. Members of the BCC possess an array of different types of appendage pili: electron microscopy studies identified five morphologically distinct classes of appendage pili in BCC strains (42).

The cable pilus is associated with the ET12 *B. cenocepacia* lineage (130) and modulates binding to host molecules such as cytokeratin 13 (116) and mucins (115) that are abundant in the CF lung. The cable pilus gene cluster is located on chromosome 2 and consists of seven CDSs (Table 3), four of which encode structural and processing components (*cblDCAB*) and three of which encode regulatory components (*cblRTS*). The pili are arranged as large peritrichous individual fibers 2 to 4 μm in length and are associated with a 22-kDa adhesion protein (AdhA) (137). Both the *cbl* cluster and *adhA* are in RODs, as is one of the three chaperone-usher-type fimbria clusters contained within the J2315 genome (Table 3). The first of these clusters encodes a fimbrial protein (BCAL1677) similar to the type I fimbrial protein FimA from *E. coli* (60). The other two clusters do not contain homologs of characterized fimbrial proteins, although both clusters contain exported proteins (BCAL1826; BCAL2634a and BCAL2635) which may be fimbrial components.

Type IV pili have been shown to modulate a variety of processes, including adhesion, twitching motility, and biofilm initiation and development (20). In *B. pseudomallei* a type IV

pilin deletion mutant (*pilA* mutant) was attenuated in mouse and nematode models of virulence (36). Type IV pili have also been shown to play a role in the adherence of *B. pseudomallei* to eukaryotic cells. Microcolony formation is a key process in cell adherence—an ability reduced in *pilA* mutants (15). In *P. aeruginosa*, type IV pili have been shown to bind to human epithelial cells (53), as well as induce apoptosis (55). The observation that these pili also bind DNA (143) suggests that they may play an important role in the formation of biofilms in the CF lung, where DNA is an abundant matrix molecule. There are several loci in the J2315 genome that encode components of a type IVa pilus, as well as two clusters that encode Flp-type pili (Table 3) (58).

The J2315 genome contains eight BuHA family proteins (Table 3), five of which are unique to J2315 and are present in RODs (see Table S2 in the supplemental material). This family of autotransporting membrane proteins contain a C-terminal YadA domain, together with HIM and Hep_Hag domains, a domain architecture that is shared with hemagglutinins and invasins that mediate bacterial interactions with host cells or extracellular matrix proteins. In *B. mallei* BuHA proteins expressed in vivo during experimental equine glanders infection were found to be immunodominant (131). The distribution of these proteins is widespread in gram-negative bacteria; however, the genomes of *Burkholderia* species, especially the pathogenic members of the genus, contain greater numbers of members of this family.

Iron metabolism. Iron is vital for life; however, much of the iron in the human body is complexed by compounds such as ferritin. In order for *B. cenocepacia* to survive in the host, iron must be scavenged via the production and uptake of siderophores. Biosynthesis clusters for ornibactin, salicylic acid (SA), and pyochelin siderophores are present in the J2315 genome (Table 3). *B. cenocepacia* produce the iron-chelating siderophores ornibactin, pyochelin, and SA in a strain-dependent manner (145). Ornibactin has been shown to be the most important of these in CF lung pathogenesis and consists of a mixture of modified tetrapeptides with three different side groups (128, 145). The ornibactin biosynthetic cluster is located on chromosome 1 (1), whereas the SA and pyochelin clusters are situated on chromosome 2 (111). The ability of J2315 to produce pyochelin is compromised since the pyochelin biosynthesis gene *pchF* (BCAM2230) contains a frameshift mutation. However, the genes encoding the transport and utilization of pyochelin in J2315 are intact and therefore probably functional.

Motility. Flagella have been shown to play an important role in the pathogenesis of *B. cenocepacia*, contributing to the invasion of lung epithelial cells (133) and modulating the immune response via the Toll-like receptor 5 (138). Five gene clusters on chromosome 1 together encode the components of a complete flagellum system (Table 3). Two duplicated components of this system are encoded on the other replicons: flagellar basal body protein FlgE2 (BCAM0987; paralog of BCAL0567) and flagellar hook-associated protein FliD2 (BCAS0104; paralog of BCAL0113). In *P. aeruginosa* two distinct flagellar hook-associated proteins have been identified (6) and shown to be antigenically distinct. In addition to being structural components of the flagella, flagellar cap proteins also bind mucin (7), an important initial event in the coloni-

zation of the CF lung. The additional copy of an antigenically distinct *fliD* therefore provides J2315 with variants, which it may use to evade the host immune system during the initial stage of infection.

Stress response. Intracellular survival of BCC bacteria within macrophages may contribute to bacterial persistence within the lung and airways of patients with CF and to sustained tissue inflammation (17, 87, 113). Resistance to oxidative stress is often associated with the ability of bacterial pathogens to survival within macrophages. Two of the most potent mechanisms utilized by activated macrophages to kill bacteria involve the production of reactive oxygen and reactive nitrogen oxide species. The detoxification of nitric oxide in *Salmonella enterica* serovar Typhimurium involves the flavohemoglobin HmpA (129), and the J2315 genome contains homologue of *hmpA* (Table 3). The detoxification of superoxide requires conversion of superoxide to hydrogen peroxide, encoded by *sod* genes, followed by destruction of the hydrogen peroxide by catalases, encoded by *kat* genes. The J2315 genome contains homologues of *sodB* (BCAL2757), *sodC* (BCAL2643), *katA* (BCAM2107), and *katB* (BCAL3299), as well as an additional catalase (BCAM0931) and a manganese-containing catalase (BCAS0635). There are also five NRAMP (natural resistance-associated macrophage protein; Table 3) family proteins in the genome. These divalent transition metal transporters are involved in iron metabolism and play a role in bacterial response to reactive oxygen species (59, 144).

Drug resistance. Strains of BCC exhibit high levels of antibiotic resistance, so much so that some BCC strains can use penicillin G as a sole carbon source (14). The drug resistances of strains infecting CF patients are often considered markers of mortality and in this way are considered virulence factors. In the BCC, resistance to multiple antibiotics is produced by multiple mechanisms that include alterations in cell permeability, the production of modifying or degradatory enzymes, and antibiotic target alteration. Other mechanisms of resistance may also be related to diminished antibiotic access (16), including drug efflux (150). J2315 is resistant to the aminoglycosides amikacin and tobramycin, the macrolide azithromycin, the β-lactams imipenem and piperacillin, and cotrimoxazole (trimethoprim-sulfamethoxazole). The strain also exhibits intermediate resistance to the fluoroquinolone ciprofloxacin.

Resistance to the β-lactam antibiotics appears to be caused by synergistic mechanisms, including the induction of chromosomal β-lactamases (109, 135) and decreased drug access (5, 104). There are at least four β-lactamases encoded in the J2315 genome, including: two class A, one class C, and one class D (Table 4). In addition, there are several β-lactamase family proteins containing β-lactamase Pfam domains (PF00144) that may have antimicrobial resistance functions.

Efflux systems can modulate broad-spectrum antibiotic resistance, as well as resistance to specific antimicrobial compounds. Multiple transport systems belonging to six families associated with drug resistance were identified in the J2315 genome: MFS (major facilitator superfamily), ABC (ATP binding cassette) family, RND (resistance nodulation division) family, MATE (multidrug and toxic compound extrusion) family, SMR (small multidrug resistance) family, and fusaric acid resistance family proteins (Table 4). Some of these families have many members in the J2315 genome; however, it is un-

TABLE 4. Potential drug resistance determinants in the genome of *B. cenocepacia* strain J2315

Gene product	CDSs[a]	Resistance	Reference
Chromosome 1			
RND family efflux transporter	BCAL1079 to BCAL1081	Unknown	94
Fusaric acid resistance family transporter	BCAL1176* to BCAL1178*	Unknown	139
Fosmidomycin resistance protein	BCAL1451	Fosmidomycin	41
Fusaric acid resistance family transporter	BCAL1453 to BCAL1456	Unknown	139
MFS efflux transporter	BCAL1510 to BCAL1512	Unknown	73
RND family efflux transporter	BCAL1674 to BCAL1676	Aminoglycosides	2, 90
RND family efflux transporter protein	BCAL1778	Unknown	
RND family efflux transporter	BCAL1811 to BCAL1813	Unknown	
MATE family multidrug resistance protein	BCAL1907	Unknown	
RND family efflux transporter	BCAL2134 to BCAL2136	Unknown	
RND family efflux transporter	BCAL2820 to BCAL2822	Unknown	77
MATE family multidrug resistance protein	BCAL2907	Polymyxin B	38
Dihydrofolate reductase	BCAL2915	Trimethoprim	18
Tetracycline resistance protein, class C (pseudogene)	BCAL3259	Tetracycline	3
MFS efflux transporter	BCAL3511 and BCAL3514	Unknown, lacks HylD family component	73
Chromosome 2			
MFS efflux transporter	BCAM0199 to BCAM0201	Unknown	
MarC family protein	BCAM0237A	Unknown	28
β-Lactamase, class D	BCAM0393	β-Lactams	107
RND family efflux transporter	BCAM0925 to BCAM0927	Aminoglycosides	78
Aminoglycoside 3′-phosphotransferase	BCAM0928*	Aminoglycosides	13
Aminoglycoside-3′′-adenylyltransferase	BCAM1013A*	Aminoglycosides	52
MFS efflux transporter protein	BCAM1132*	Unknown	
MFS efflux transporter protein	BCAM1202	Unknown	
MFS efflux transporter protein (pseudogene)	BCAM1251	Unknown	
RND family efflux transporter	BCAM1419 to BCAM1421	Unknown	
Fusaric acid resistance family transporter protein	BCAM1452	Unknown	139
MarC family protein	BCAM1709	Unknown	28
Fusaric acid resistance family transporter protein	BCAM1758	Unknown	139
MFS efflux transporter protein	BCAM1760	Unknown	
β-Lactamase, class A	BCAM1779	Ceftazidime, clavulanic acid	136
Putative multidrug efflux system transporter	BCAM1864 to BCAM1866	Unknown	
RND family efflux transporter	BCAM1945 to BCAM1947	Olaquindox	48
β-Lactamase, class A	BCAM2165	β-Lactams	135
Macrolide-specific efflux system transporter	BCAM2186 to BCAM2188	Macrolides	61
MFS efflux transporter	BCAM2334, BCAM2335, and BCAM2337	Unknown	
Rifampin ADP-ribosyl transferase	BCAM2385	Rifampin	110
RND family efflux transporter	BCAM2549 to BCAM2551	Chloramphenicol, trimethoprim, and ciprofloxacin	19, 95
Streptomycin 3′-kinase	BCAM2633	Aminoglycosides	88
Chromosome 3			
Fusaric acid resistance family transporter protein	BCAS0014 to BCAS0017	Unknown	139
β-Lactamase, class C	BCAS0156	β-Lactams	85
MFS efflux transporter protein	BCAS0289	Unknown	
Putative glutathione transferase	BCAS0336*	fosfomycin	96
MFS efflux transporter	BCAS0471 to BCAS0473	Unknown	
RND family efflux system transporter	BCAS0582 to BCAS0584	Unknown	
RND family efflux system transporter	BCAS0591 to BCAS0593	Unknown	
RND family efflux system transporter	BCAS0764 to BCAS0766	Aminoglycosides gentamicin and streptomycin, macrolide erythromycin	24, 47

[a] An asterisk (*) indicates a gene(s) is associated with that function but absent from both *B. cenocepacia* AU1054 and HI2424 genomes.

clear from in silico analysis alone whether or not they play a role in antibiotic resistance. For example, 16 CDSs were identified in the J2315 genome that encode efflux pumps belonging to the RND family. Two of these CDSs belong to systems that

have been shown to be associated with drug resistance in *B. cenocepacia*: BCAM2550 (*ceoB*) is a component of a system that encodes chloramphenicol, trimethoprim, and ciprofloxacin resistance (19, 95), and BCAS0765 is associated with resis-

tance to the antibiotics fluoroquinolones, tetraphenylphosphonium, and streptomycin, as well as to ethidium bromide (47). In addition, the genome contains orthologues of RND efflux proteins that have been shown to mediate resistance to antibiotics (2, 77, 78, 90, 94), metals (39, 66, 103), and other antimicrobial compounds (48) in other organisms.

Comparisons with other sequenced strains show that the J2315 genome contains strain-specific CDSs (Table 4) that may contribute to its elevated drug resistance, for example: a putative fusaric acid efflux system, RND family efflux systems, an aminoglycoside 3′-phosphotransferase, and a multiple antibiotic resistance protein.

Evolution of virulence in the ET12 lineage. Considering the pathogenic pedigree of J2315, it was surprising that several virulence determinants that have been shown to be important for *B. cenocepacia* pathogenicity were pseudogenes in J2315 (Table 5). In order to discover how widely distributed these mutations were we screened five of the virulence factor pseudogene loci in *B. cenocepacia* strains. Multilocus sequence typing was used to select strains as it proved additional resolution for distinguishing strains within, and related to, the ET12 lineage (Table 6) (9). Four strains belonging to the same sequence type (ST) as J2315 (ST28) were screened, along with two other closely related strains (BCC0016 and K56-2) that are single and double locus variants of ST28 (ST29 and ST30, respectively; http://pubmlst.org/bcc).

Screening of the virulence pseudogenes revealed the likely relative timescales of acquisition of these mutations. For example, pseudogenes that disrupt pyochelin biosynthesis and cepacian capsule functions were identified in all of the ET12 strains tested (Table 6), suggesting that they occurred in an ancestral strain, whereas the O-antigen cluster, T2SS, and uncharacterized EPS cluster pseudogenes were intermittently distributed, indicating that they are recent mutational events.

The observation of independent mutations in the ET12 strain K56-2 uncharacterized EPS CDS suggests ongoing selection for the loss of this potential virulence function in the CF lung. There is further evidence for pathoadaptation involving exopolysaccharide structures in the PHDC lineage of *B. cenocepacia* (i.e., strains AU1054 and HI2424). In the *B. cenocepacia* IIIB strains there are divergent clusters at orthologous loci for the LPS O antigen and EPS. In AU1054, both of these clusters are disrupted by IS element insertions, whereas the HI2424 clusters remain intact.

The modification of core functions via point mutation has also contributed to drug resistance in J2315. Trimethoprim interferes with the action of bacterial dihydrofolate reductase (DfrA), inhibiting synthesis of the essential tetrahydrofolic acid. Members of the ET12 lineage exhibit different sensitivities to trimethoprim (100). To investigate the evolution of trimethoprim resistance in the ET12 lineage, we sequenced *dfrA* from members of this clonal group that have different trimethoprim MICs (K56-2 and BCC0179, MIC < 2 mg/liter; J2315, BCC0016, and BC7, MIC > 32 mg/liter). J2315, BCC0016, and BC7 all contain a single nonsynonymous nucleotide substitution at codon 99 (CTC to ATC), resulting in a leucine-for-isoleucine substitution. In an experiment with *E. coli* mutator strains exposed to antibiotics, resistance to trimethoprim was shown to be the result of a single point muta-

tion in DfrA, Ile94 to Leu, for which the equivalent residue in *B. cenocepacia* is exactly Ile99/Leu99 (89).

DISCUSSION

B. cenocepacia is a versatile environmental organism that has emerged as an important pathogen of CF patients. Using the J2315 genome we have been able to investigate the genomic basis for the success of this CF pathogen and examine the evolutionary mechanisms that may lead to its emergence and ongoing spread.

Comparative analysis of *Burkholderia* genomes reveals that horizontal gene transfer has contributed to the genomic plasticity of this versatile group of organisms. The exchange of MGEs and movement of genomic islands facilitates the spread of genes between genetically diverse bacteria, a process which could be advantageous to the bacterium in its existing environment or allow adaptation to new niches, such as the CF lung. The J2315 genome contains 14 genomic islands that are absent from the other *B. cenocepacia* strains. Some of the islands share similarity with islands in other *Burkholderia* spp., suggesting that the extent of the *B. cenocepacia* pan genome extends well beyond that of the species. The acquisition of genomic islands appears to have been seminal in the evolution of the ET12 lineage, introducing functions that promote survival and pathogenesis in the CF lung. One such island is the CCI (BcenGI11). This island plays a role in infection, is ubiquitous in the ET12 lineage, and is more common in *B. cenocepacia* IIIA strains than IIIB (10, 84). The contribution of the other genomic islands to the virulence and survival of J2315 in the CF lung remains to be resolved, since many of the functions encoded in the genomic islands are associated with enhancing the metabolic repertoire of the bacterium or are unknown.

Evidence of the pathogenic specialization of the ET12 lineage can be found in the other RODs. These regions do not appear to have the properties of MGEs and as such represent more stable components of the J2315 genome, albeit some may have arisen by horizontal gene transfer in the more distant past. Contained within this unique component of the J2315 genome are the cable pilus locus and the 22-kDa adhesion protein AdhA. These proteins bind cytokeratin 13 (116), a cytoplasmic protein that may become surface exposed during the course of chronic infection in CF (114), and also mucins (115), which are produced in abundance in the CF lung due to poor clearance. The cable pilus/AdhA complex is also associated with the ability of *B. cenocepacia* to bind to CF lung explant tissue (114) and bind and invade epithelial cells (117). Intriguingly, the CDSs encoding these components are at separate loci on chromosome 2, and orthologs are absent from the other BCC strains examined. This suggests that the pilus and the 22-kDa adhesin may have independent origins, but their concurrence in J2315 has resulted in functional synergy. Other virulence functions found within the J2315-specific RODs include surface polysaccharide biosynthesis, BuHA family putative adhesins, chaperone-usher type fimbriae, and a phospholipase C.

In recent years *B. cenocepacia* strains have acquired additional resistances to antibiotics commonly used in the treatment of CF patients. In particular, strains from within the

TABLE 5. Pseudogenes in the genome of *B. cenocepacia* J2315

Chromosome and gene product	CDSs	Mutation type
Chromosome 1		
Gly/Ala/Ser-rich lipoprotein	BCAL0138	Nonsense (amber)
Type I modification component of restriction-modification system	BCAL0414	Disruption, IS element
Putative transferase	BCAL0648	Frameshift
Putatve PTS system, EIIA 2 component	BCAL0810	Nonsense (amber)
UvrABC system protein C 2	BCAL1014	Disruption, IS element
Putative transposase	BCAL1028	Nonsense (amber) and frameshift
IclR family regulatory protein	BCAL1082	Nonsense (ochre)
Conserved hypothetical protein	BCAL1124a	Disruption, IS element
Conserved hypothetical protein	BCAL1132	Disruption, IS element
Conserved hypothetical protein	BCAL1169	Disruption, IS element
Conserved hypothetical protein	BCAL1302	Disruption, IS element
Putative SNF2-related helicase	BCAL1308	Disruptions, IS elements
Putative oxidoreductase	BCAL1558	Disruption, prophage
TetR family regulatory protein	BCAL1672	Frameshift
TonB-dependent receptor	BCAL1783	Nonsense (amber)
Putative DNA-binding protein	BCAL2066	Frameshift
Hypothetical protein	BCAL2219	Disruption, IS element
Conserved hypothetical protein	BCAL2279	Disruption, IS element
Putative ATP-dependent DNA helicase	BCAL2480	Disruption, IS element
Putative transposase	BCAL2496	Disruption, IS element
Putative methionine aminopeptidase	BCAL2533	Disruption, IS element
Conserved hypothetical protein	BCAL2574	Nonsense (amber)
Putative transposase-related protein	BCAL2581	Frameshift
Conserved hypothetical protein	BCAL2591	Disruption, IS element
ABC transporter ATP-binding protein	BCAL2601	Frameshift and deletion
UvrABC system protein C1	BCAL2854	Disruption, IS element
Glycosyltransferase	BCAL3125	Disruption, IS element
Putative capsule polysaccharide biosynthesis/export protein	BCAL3223	Disruption, IS element
Putative transposase	BCAL3248	Disruption, IS element
Putative transposase	BCAL3249	Disruption, IS element
Tetracycline resistance protein, class C	BCAL3259	Frameshift
Glutamate/aspartate ABC transporter ATP-binding protein GltL	BCAL3354	Frameshift
General secretory pathway protein L	BCAL3517	Frameshift
Chromosome 2		
Putative pyridoxal-dependent decarboxylase	BCAM0245	Disruption, IS element
Conserved hypothetical protein	BCAM0274a	Frameshift
Putative conjugal transfer protein	BCAM0335	Frameshift
Putative acetyltransferase–GNAT family	BCAM0354a	Frameshifts
AraC family regulatory protein	BCAM0454	Frameshift
Conserved hypothetical protein	BCAM0520	Disruption, IS element
TonB-dependent receptor	BCAM0706	Frameshift
Conserved hypothetical protein	BCAM0788	Frameshift
Undecaprenyl-phosphate glucosyl-1-phosphate transferase	BCAM0856	Frameshift
Major Facilitator Superfamily protein	BCAM1102	Nonsense (amber)
Putative multidrug resistance transporter	BCAM1251	Disruption, IS element
Conserved hypothetical protein	BCAM1328	Frameshifts
Amino acid permease	BCAM1498	Frameshift
Putative short-chain dehydrogenase	BCAM1794	Nonsense (amber)
Putative GTP cyclohydrolase II	BCAM1874	Disruption, prophage
Hypothetical phage protein	BCAM1914	Disruption, IS element
Phage integrase	BCAM1923	Disruption, IS element
Putative plasmid stability protein	BCAM2006a	Frameshift
Putative plasmid stability protein	BCAM2006b	Frameshift
Conserved hypothetical protein	BCAM2043	Frameshift
Conserved hypothetical	BCAM2101	Disruption, IS element
Putative pyochelin synthetase PchF	BCAM2228	Frameshift
Putative porin-related protein	BCAM2621	Nonsense (opal)
Two-component regulatory system, response regulator protein	BCAM2837	Frameshift
Chromosome 3		
Conserved hypothetical protein	BCAS0180	Frameshift
ABC transporter ATP-binding protein	BCAS0221	Frameshift
ABC transporter ATP-binding protein	BCAS0421	Disruption, IS element
cis-1,2-Dihydroxycyclohexa-3,5-diene-1-carboxylate dehydrogenase	BCAS0493	Frameshift
Putative short chain dehydrogenase	BCAS0644	Frameshifts
Putative short chain dehydrogenase	BCAS0645	Frameshifts
LysR family regulatory protein	BCAS0646	Frameshift
Putative transposase	BCAS0651	Disruption, IS element
Putative transposase	BCAS0681	Disruption, IS element
Plasmid		
Putative hemagglutinin-related autotransporter protein	pBCA011	Frameshift

ET12 lineage have different sensitivities to ciprofloxacin, tobramycin, tetracycline, and trimethoprim (100). In comparison to other members of the ET12 lineage, J2315 has developed enhanced resistance to a number of antibiotics (100). Indeed,

we found that the J2315 genome contains drug resistance in genomic islands and RODs, highlighting the role that horizontal gene transfer has played in the evolution of drug resistance in even the most intrinsically resistant of organisms. The ge-

TABLE 6. Distribution of virulence factor pseudogenes in *B. cenocepacia* IIIA strains[a]

| Strain | MLST | Distribution of virulence factor pseudogenes for (strain, factor): | | | | |
		BCAL3125§, O antigen	BCAL3223§, uncharacterized EPS	BCAL3517†, T2SS	BCAM0856†, cepacian capsule	BCAM2228†, pyochelin siderophore
J2315*	ST28	ISBcen20 interrupted†	IS*407* interrupted†	110-bp deletion	11-bp deletion	Frameshift
BCC0077*	ST28	Uninterrupted	Uninterrupted	In frame	11-bp deletion	Frameshift
BCC0179*	ST28	Uninterrupted	Uninterrupted	In frame	11-bp deletion	Frameshift
BCC0162*	ST28	Uninterrupted	Uninterrupted	In frame	11-bp deletion	Frameshift
BCC0313*	ST28	Uninterrupted	Uninterrupted	In frame	11-bp deletion	Frameshift
BCC0016*	ST29	As J2315†	Uninterrupted	In frame	11-bp deletion	Frameshift
K56-2*	ST30	Uninterrupted	ISBcen13 interrupted†	In frame	11-bp deletion	Frameshift
BCC1261	ST201	No product	Uninterrupted	In frame	In frame	In frame
BCC0222	ST234	No product	Uninterrupted	In frame	In frame	In frame

[a] Multilocus sequence typing (MLST) was used to identify strains related to J2315. The majority of the strains screened belonged to the ET12 lineage (an asterisk [*] indicates members of the ET12 lineage). Included in the screening were two non-ET12 strains, BCC1261 (ST201) and BCC0222 (ST234), that are more distantly related to the strains that belong to ET12 but are members of the same clonal complex as defined by MLST (9) and therefore may be representative of the ancestral genotype. Where indicated, mutations were determined by sequence data (†) or PCR (§).

nome also provides evidence for the evolution of drug resistance through point mutation, elucidating a nonsynonymous base change in the dihydrofolate reductase gene that generates trimethoprim resistance.

Although the success of J2315 may be in part due to the acquisition of new functions, gene loss via mutation appears to have also played an important role. J2315 is a formidable pathogen of the CF lung; once infected with ET12, the life expectancy of a patient shortens dramatically (57). It is therefore surprising that the J2315 genome contains pseudogenes, formed via both IS disruption and frameshift mutations, in important *B. cenocepacia* virulence functions, such as O antigen and capsule (Table 6). Many of the putative virulence determinants identified in J2315 are shared with other BCC strains. These functions may therefore have important roles for the survival of BCC in its natural reservoir rather than in an opportunistic pathogen niche. In the case of J2315, the emergence and patient-to-patient spread of ET12 may mean that many of the functions required for survival in the environment are no longer required and have become superfluous or even disadvantageous. The level of pseudogenes and partial genes in the J2315 genome (1.7% of CDSs) is similar to the level found in most other bacterial genomes (72), suggesting that there is not an elevated level of mutation in this strain.

The screening of the virulence pseudogenes in other ET12 strains showed that some of the J2315 mutations may have occurred early on in the evolution of the ET12 lineage, whereas others represent recent strain-specific mutations. Some of these mutations may therefore represent formative pathoadaptive mutations that contributed to the initial success and emergence of the ET12 lineage, whereas others may be indicative of the ongoing selection pressures in the CF lung.

All of the ET12 strains that we screened contained the same frameshift mutation in the pyochelin siderophore biosynthesis gene *pchF*. Interestingly, the siderophores produced by CF isolates of *B. cenocepacia* exhibit strain variation, with SA and ornibactins being the most prevalent, followed by pyochelin (32). In a study that investigated pyochelin production in CF patients from Toronto and Cleveland (125), pyochelin-negative strains were isolated from patients with moderate or mild

infections, whereas pyochelin-positive strains were more frequently isolated from patients with severe pulmonary disease going on to suffer high mortality. It is possible that pyochelin production may play an important role in the progress of *B. cenocepacia* disease in CF patients. Switching off expression of the pyochelin production in ET12 strains may promote persistence in the CF lung and thus the spread of the members of this lineage between patients.

The long-term maintenance of infection in the CF lung may result in the streamlining of a pathogen's virulence and drug resistance functions, since functions required for the initiation of acute infections may be selected against during chronic infections. Evidence for recent pathoadaptive mutations came from the observation of an independent mutation in a glycosyltransferase of an uncharacterized EPS cluster, in K56-2, another member of the ET12 lineage. Further evidence for pathoadaptation involving surface carbohydrates came from the in silico comparison of the *B. cenocepacia* strains; in the CF epidemic strain there are mutations in the LPS O-antigen cluster and an EPS cluster, whereas the related environmental strain's clusters remain intact. A reduction in glycosylated surface molecules may provide some advantage, such as reducing immunorecognition in the lung, thus promoting the maintenance of a long-term infection. In a study of the EPS production in a collection of 506 *B. cenocepacia* strains isolated from CF patients in the Vancouver area over a 26-year period, more than half were nonmucoid (151). The study also revealed evidence of phenotype switching in sequential isolates from individual patients, with the conversion from mucoid to nonmucoid being the most prevalent switch. The authors hypothesized that the loss of EPS may reflect adaptation from persistence in the CF lung to increased disease severity.

Evidence for pathoadaptation can also be found in *P. aeruginosa*, the major pathogen of the CF lung, where the loss of acute virulence determinants has been observed in CF isolates, suggesting that these products are dispensable for long-term maintenance of *P. aeruginosa* in vivo (76, 147). A recent study by Smith et al. investigated genetic adaptation of *P. aeruginosa* in CF infections (123). Genomic sequencing of strains isolated from a CF patient 8 years apart, as well as additional chronic

infections, identified that virulence factors genes were the most prevalent class of genes mutated during the course of infections. Significantly one of the *P. aeruginosa* virulence functions that acquired deleterious mutations was the O antigen (123), a function also lost in J2315 (101). One additional virulence mutational adaptation is also shared: *mexZ*, a negative regulator of the *mexXY* component of the MexXY-OprM multidrug-efflux pump, is orthologous to a J2315 pseudogene (BCAL1672). In *P. aeruginosa*, upregulation of this multidrug-efflux pump is associated with resistance to aminoglycoside antibiotics that are routinely used to treat infection in CF patients (124).

Although there are parallels in the potential pathoadaptations of *P. aeruginosa* and *B. cenocepacia*, there are also intriguing differences. The high frequency of nonmucoid *B. cenocepacia* isolated from CF patients (151) is in marked contrast to *P. aeruginosa*, where isolates from CF patients are more frequently mucoid than nonmucoid (45). In *P. aeruginosa* the production of the EPS alginate is linked to increased morbidity and mortality (45), whereas strains of *B. cenocepacia* that are considered to be more virulent, such as those in the ET12 lineage, have been shown not produce EPS (11, 151). These somewhat paradoxical observations point toward subtle differences in the role that the different EPS plays in the mechanism of pathogenicity and host-cell interaction in these two CF pathogens.

The genome sequence of J2315 has afforded a tantalizing glimpse of components of the genome that may promote growth in the CF lung and provided clues to the potency and spread of ET12 in recent decades. Evidence from comparative genomics suggests that loss of functions through mutation and gain of functions via horizontal gene transfer appear to promote growth and persistence in the CF lung and contribute to the success of J2315. Much remains to be learned, however, as the pathology of *B. cenocepacia* infections and the physiology of the CF lung are both complex. The complete genome sequence will therefore be a valuable resource for future investigation into disease caused by *B. cenocepacia*.

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AUTHOR'S CORRECTION

The Genome of *Burkholderia cenocepacia* J2315, an Epidemic Pathogen of Cystic Fibrosis Patients

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