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The *Burkholderia cepacia* Epidemic Strain Marker Is Part of a Novel Genomic Island Encoding Both Virulence and Metabolism-Associated Genes in *Burkholderia cenocepacia*

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The *Burkholderia cepacia* epidemic strain marker (BCESM) is a useful epidemiological marker for virulent *B. cenocepacia* strains that infect patients with cystic fibrosis. However, there was no evidence that the original marker, identified by random amplified polymorphic DNA fingerprinting, contributed to pathogenicity. Here we demonstrate that the BCESM is part of a novel genomic island encoding genes linked to both virulence and metabolism. The BCESM was present on a 31.7-kb low-GC-content island that encoded 35 predicted coding sequences (CDSs): an *N*-acyl homoserine lactone (AHL) synthase gene (*cciI*) and corresponding transcriptional regulator (*cciR*), representing the first time cell signaling genes have been found on a genomic island; fatty acid biosynthesis genes; an IS*66* family transposase; transcriptional regulator CDSs; amino acid metabolism genes; and a group of hypothetical genes. Mutagenesis of the AHL synthase, amidase (*amiI*), and porin (*opcI*) genes on the island was carried out. Testing of the isogenic mutants in a rat model of chronic lung infection demonstrated that the amidase played a role in persistence, while the AHL synthase and porin were both involved in virulence. The island, designated the *B. cenocepacia* island (cci), is the first genomic island to be defined in the *B. cepacia* complex and its discovery validates the original epidemiological correlation of the BCESM with virulent CF strains. The features of the cci, which overlap both pathogenicity and metabolism, expand the concept of bacterial pathogenicity islands and illustrate the diversity of accessory functions that can be acquired by lateral gene transfer in bacteria.

The *Burkholderia cepacia* complex (BCC) is a group of closely related species which inhabit diverse niches from the natural environment (30) to human opportunistic infection (21). Individuals with cystic fibrosis (CF) are particularly prone to BCC infection, with patient-to-patient spread (21) and high rates of morbidity and mortality having been documented (28). Originally known as the single species *B. cepacia*, these bacteria have been recently reclassified into distinct genetic species or genomovars and currently comprise eight new species and one genomovar (6, 21): *B. cepacia* (which will remain as the formal name for genomovar I, as it contains the species type strain); *B. multivorans* (genomovar II); *B. cenocepacia*, the new name for genomovar III (45); *B. stabilis* (genomovar IV); *B. vietnamiensis* (genomovar V); *B. cepacia* genomovar VI, which has not been formally named; *B. ambifaria* (genomovar VII); *B. anthina* (genomovar VIII [44]), and *B. pyrrocinia* (genomovar IX; 44). All these genomovars or species have been recovered from patients with CF infection (6, 21, 44, 45); however, the relative virulence and pathogenicity of each BCC species has not been fully determined.

The prevalence of infection in several CF populations has shown that *B. cenocepacia* is the most predominant BCC pathogen, causing on average 67% of cases of BCC infection (21). *B. multivorans* is the second most prevalent species, causing between 5 and 37% of cases dependent on the CF population examined, and the remaining genomovars constitute around 5% (21). In addition to its dominance as a CF pathogen, *B. cenocepacia* has also been associated with the majority of well-documented cases of patient-to-patient spread (11, 25, 45). Epidemiological data have demonstrated that it is highly virulent and capable of replacing infection with *B. multivorans* (28) and causing significant mortality among both CF patients (21) and those which have undergone lung transplantation (2). These data from cases of clinical infection suggest that *B. cenocepacia* may encode more-potent or larger numbers of virulence factors; yet, despite these advances in our understanding of the taxonomy and epidemiology of the complex, knowledge of specific virulence mechanisms is not extensive. Virulence factors characterized in *B. cenocepacia* strains and known to play a role in vivo include epithelial cell and mucin binding mediated by the cable pilus (35); a quorum sensing pathway mediated by classical LuxRI homologs, CepRI (17); iron acquisition and the production of siderophores (37); and cellular invasion and intracellular survival mechanisms (29), including a type III secretion system (42). Many of these virulence factors have homologs in other pathogens and none appear to be unique to *B. cenocepacia*.

The *B. cepacia* epidemic strain marker is a unique DNA region originally identified in strains of "*B. cepacia*" which had

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Feature(s)	Reference or source
Strains		
B. cenocepacia J2315	CF strain; type strain for species and genome sequence strain	25, 45
B. cenocepacia K56-2	CF strain and experimental strain used for construction of site-directed gene mutants and infection studies	17, 37
B. cenocepacia CEP54B	CF strain representative of Cleveland epidemic strain type 17	25
B. cenocepacia CEP54A	Derivative of CF strain CEP54B which lacks BCESM region	This study
BCC strains	Selected from published strain panels and Cardiff University collection to be representative of all current genomovars	7, 22, 25, 40
E. coli JM109	Standard cloning strain	36
K56-2*cciI*	AHL synthase knockout mutant disrupted at single *Bsm*I site (position 235 in 654-bp CDS)	This study
K56-2*amiI*	Amidase knockout mutant disrupted at single *Cla*I site (position 233 in 1,401-bp CDS)	This study
K56-2*opcI*	Porin knockout mutant disrupted at single *Bam*HI site (position 734 in 1,001-bp CDS)	This study
Plasmids		
pGEM-T	PCR product cloning vector	Promega Corporation Inc.
pUC-TP	1.1-kb trimethoprim resistance cassette	37
pJQ200SK	Gentamicin-resistant, *sacB*, Mob$^+$, P15A replicon	32
pEGM105-Tc	pJQ2000SK with tetracycline resistance cassette inserted into *Bgl*I site within Gm^r gene; suicide vector used for allelic exchange mutagenesis	Kindly provided by G. Mendrano
pCciI-Tp	pEGM105-Tc carrying AHL synthase gene interrupted at *Bsm*I site with Tp cassette	This study
pAmiI-Tp	pEGM105-Tc carrying amidase gene interupted at *Cla*I site with Tp cassette	This study
pOpcI-Tp	pEGM105-Tc carrying porin gene interupted at *Bam*HI with Tp cassette	This study

spread from patient-to-patient in CF (27). It has been widely applied in infection control as a clinical risk marker (21, 28, 40). The *B. cepacia* epidemic strain marker (BCESM) was identified during random amplified polymorphic DNA (RAPD) typing of "*B. cepacia*" isolates as a conserved amplification product in otherwise-distinct strain fingerprints (27). The 1.4-kb BCESM DNA encoded a single CDS for a putative negative transcriptional regulator designated *esmR* (27). Subsequent analysis demonstrated that the marker was exclusive to strains of *B. cepacia* genomovar III (22), now known as *B. cenocepacia* (45). Phylogenetic analysis of the *recA* gene of *B. cenocepacia* indicated that there were at least two distinct strain lineages within the species, III-A and III-B (22). Epidemiological studies have demonstrated that the BCESM is present in more than 77% of III-A CF strains but is absent in the majority of III-B CF strains (19, 40). The BCESM does not appear to be an absolute marker for the ability of *B. cenocepacia* strains to cause CF infection and is also not associated with all strains which infect multiple patients (18). However, in CF populations where BCESM positive strains are prevalent, they have been associated with well-documented transmissibility, virulence, and mortality (28, 40). In addition, the major *B. cenocepacia* clone which infects the majority of CF patients in Canada and the United Kingdom, known as the ET12 lineage (15) or cable pilus strain (41), is unique in being the only *B. cenocepacia* strain identified to encode both the cable pilus virulence factor and BCESM (21). However, unlike the cable pilus, no virulence function has been associated with the BCESM, despite its correlation to clinically problematic strains. Here we report further characterization of the *B. cepacia* epidemic strain marker locus using DNA sequence from the *B. cenocepacia* genome project and gene mutagenesis to determine the role of BCESM encoded genes during infection. Evidence that the BCESM is part of a novel *B. cenocepacia* genomic island is described.

MATERIALS AND METHODS

Bacterial isolates, culture, strain typing, and general reagents. The bacterial strains and plasmids used are shown in Table 1. Genome sequence data were obtained for *B. cenocepacia* strain J2315 and all genetic manipulation was performed in the clonally identical *B. cenocepacia* strain, K56-2 (Table 1) (25). Other BCC strains were drawn from the Cardiff University collection or previously published studies and cultured as described (7, 22, 24, 25, 28, 40). For genetic selection, *B. cenocepacia* strain K56-2 was plated onto Tryptone Soya Agar containing antibiotics as needed: trimethoprim (200 μg/ml) or tetracycline (300 μg/ml). *Escherichia coli* cloning strains were grown and selected for with antibiotics as needed (36). Genomic DNA was extracted as previously described (23), and BCC taxonomic identification was performed by analysis of the *recA* gene (22). Isolates were typed using RAPD analysis as the primary screen (24) and pulsed-field gel electrophoresis (PFGE) followed by macrorestriction with *Spe*I as the secondary strain identification method (25, 46). All chemicals were obtained from Sigma-Aldrich Company Ltd., Poole, Dorset, United Kingdom, unless otherwise stated. Restriction enzymes were purchased from Promega Corporation Inc. (Southampton, United Kingdom) and molecular size markers from MBI Fermentas, Inc. (distributed by Helena Biosciences, Sunderland, United Kingdom).

Nucleotide sequence and bioinformatic analysis. *B. cenocepacia* strain J2315 DNA sequence data were produced by the Pathogen Sequencing Group at the Sanger Institute, Hinxton, Cambridge, United Kingdom, and can be obtained from http://www.sanger.ac.uk/Projects/B_cenocepacia/. Bioinformatic analysis and annotation was performed using the software Artemis (34) and the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/index.html). Protein family information was obtained from the Protein Families databases of Alignments and HMMs (PFAM; http://www.sanger.ac.uk/Software/Pfam/index.shtml) and the Clusters of Orthologous Groups database (http://www.ncbi.nlm.nih.gov/COG/). Predicted coding sequences were designated numerically with the prefix BCAM (an abbreviation for *B. cenocepacia* annotation medium, because the sequence data were from the medium-sized chromosome). Restriction mapping, dot-plot analysis and preparation of figures were performed using DS Gene software (Accelerys Ltd., Cambridge, United Kingdom).

PCR and probe labeling. PCR was performed using *Taq* DNA polymerase (Qiagen, Crawley, United Kingdom) under the conditions described by the manufacturer with 20 ng of template DNA and 10 pmol of each primer added to a standard 25-μl reaction mixture (22). Thermal cycling was performed on a Flexigene Thermal Cycler (Techne Ltd., Newcastle, United Kingdom) and conditions for all primer sets were as follows: an initial cycle of 94°C for 5 min followed by 30 cycles of 94°C for 30 s, annealing at the appropriate temperature (Table 2 or noted below) for 30 s, and extension at 72°C for 60 s, with a final 5-min extension at 72°C. Southern hybridization probes were prepared by PCR in the presence of the nonradioactive label digoxigenin (DIG)-UTP as described by the manufacturer (Roche Diagnostics Ltd., Lewes, United Kingdom). Products were analyzed and sized by agarose gel electrophoresis as described previously (36).

BCC genomic array preparation. Genomic DNAs from 241 genetically distinct BCC strains were immobilized onto replicate nylon membranes for use in Southern hybridization analysis. Species represented on the array were as follows: *B. cepacia* genomovar I (23 strains), *B. multivorans* (53 strains), *B. cenocepacia* (88 strains), *B. stabilis* (12 strains), *B. vietnamiensis* (21 strains), *B. cepacia* genomovar VI (2 strains), *B. ambifaria* (19 strains), *B. anthina* (10 strains), and *B. pyrrocinia* (13 strains). Genomic DNA from each strain (at a concentration of approximately 150 ng/μl) was aliquoted into 384 well-plates (ABgene Ltd., Epsom, United Kingdom) and spotted onto replicate positively charged nylon membranes (Roche Diagnostics Ltd.), in a two-by-two pattern for each sample, using a Genomic Solutions Flexys robotic microarrayer. Membranes were placed in denaturing solution (0.5 M NaOH) for 5 min, neutralized in 1.5 M NaCl–1.5 M Tris-HCl (pH 8.0) for 5 min, and then washed for 2 min in 2× sodium chloride-sodium citrate buffer (SSC) (36). DNA was fixed to the membranes by exposure to UV light (360 nm) for 3 min.

Southern hybridization analysis. DNA separated by standard agarose gel electrophoresis or PFGE was transferred to positively charged nylon membranes using a conventional Southern transfer method (36); membranes were then denatured, neutralized and washed as described above for the genomic DNA arrays. All membranes were prehybridized for 2 h at 50°C in EasyHyb buffer (Roche Diagnostics Ltd.) prior to addition of 5 to 10 ng of heat-denatured DIG-labeled PCR probe (Table 2); the probes were then allowed to hybridize overnight at the same temperature. Washing was performed at high stringency with a final 10-min wash in 0.1× SSC containing 0.1% sodium dodecyl sulfate at a temperature of 68°C (36). Chemiluminescent detection of DIG probes was performed using the substrate CSPD in accordance with the manufacturer's protocols (Roche Diagnostics Ltd.) and membranes exposed to autoradiography film (Sigma-Aldrich Ltd.) at 37°C for up to 18 h until clear signals were observable. Control hybridizations to the genomic DNA arrays were performed using a 16S rRNA gene probe (22) amplified from pooled DNA representative of the BCC.

Site-directed gene mutagenesis. PCR probes designed from the *B. cenocepacia* J2315 sequence data were used to clone each target for subsequent gene mutagenesis. The AHL synthase gene, *cciI*, was amplified with a primer pair encoding terminal *Sma*I sites, 5′-CCCGGGACGCGCTTGATA-3′ and 5′-CCCG GGAAGCGGTGTTTG-3′, which produced the predicted 748-bp product at an annealing temperature of 56°C. The porin gene, *opcI*, was also amplified with *Sma*I-encoding PCR primers, 5′-CCCGGGCGATCGATTCAA-3′ and 5′-CCG GGGAGCTTCCAGTCC-3′, which produced the expected product of 988 bp at an annealing temperature of 56°C. The amidase gene was amplified using the primers shown in Table 2. All PCR products were then cloned into pGEM-T as described by the manufacturer (Promega Corporation Inc.). Single restriction sites were identified in the *cciI*, *amiI*, and *opcI* CDSs (*Bsm*I, *Cla*I, and *Bam*HI, respectively [Table 1]). PCR primers encoding each of these restriction sites as overhanging DNA were designed to amplify the trimethoprim resistance cassette of pUC-TP (Table 1) and the resulting 1.1-kb trimethoprim cassette PCR products cloned into pGEM-T. The appropriate trimethoprim cassette constructs were digested with *Bsm*I, *Cla*I, and *Bam*HI, respectively, and cloned into the corresponding restriction sites of the *cciI*, *amiI*, and *opcI* pGEM-T constructs. The resulting clones were analyzed by PCR with the appropriate primers shown in Table 2 to identify each correctly interrupted gene construct. Mobilizable suicide constructs were then constructed in pEGM105-Tc, a derivative of plasmid pJQ2000SK (32) encoding tetracycline resistance that was kindly provided by G. Mendrano, Texas A&M University, College Station (Table 1). The *cciI* and *opcI* mutated constructs were digested with *Sma*I and cloned into *Sma*I-linearized pEGM105-Tc, generating suicide constructs pCciI-Tp and pOpcI-Tp (Table 1). The *amiI* pGEM-T construct insert was amplified by PCR and the product blunted by incubation with Klenow polymerase as described by the manufacturer (Promega Corporation Inc.) and also cloned into *Sma*I-digested pEGM105-Tc to generate plasmid pAmiI-Tp (Table 1). Each suicide mutagenesis construct was

TABLE 2. Gene probes used to map the cci by Southern hybridization

Gene probe name	Putative gene product	PCR primer sequence (5′ to 3′)		PCR annealing temp (°C)	Product size (bp)
		Forward	Reverse		
BCAM0236	Arsenic resistance protein	GGT TCC TGC TGC TGT ACG	TCC TTG CGC TCG CTA TAC	54	574
cciI	AHL synthase	TTG ATC GCG GTC GTA CGA	ACC GCA ACG CTG GTA AAG	50	319
BCAM0248	Putative IS transposase	GCC GGA AAT CGG AGA AAC	CGT GAT GCT ACC CAT CCA	53	512
BCESM	Original RAPD marker spanning the *esmR* gene	CCA CGG ACG TGA CTA ACA	CGT CCA TCC GAA CAC GAT	62	1,418
amiI	Putative amidase	GCT TTC TTG CGC GCT ACA	ATG CAC CAC GAT CTT TGC	52	1,821
opcI	Probable porin protein	GCC TTT GTC GGA CTG AGC	GTC TGT GCG TTT CGA GAG	54	612
BCAM0278	Putative stress protein	GAT CGA CGT CAC CGA AAA	GAT CGC GAG CTT CTT CTG	52	289
BCAM0280	Hypothetical protein	CGT CGA GGA TGA ACT GGA	CCT TAC CGG ACA ACG TGA	52	317

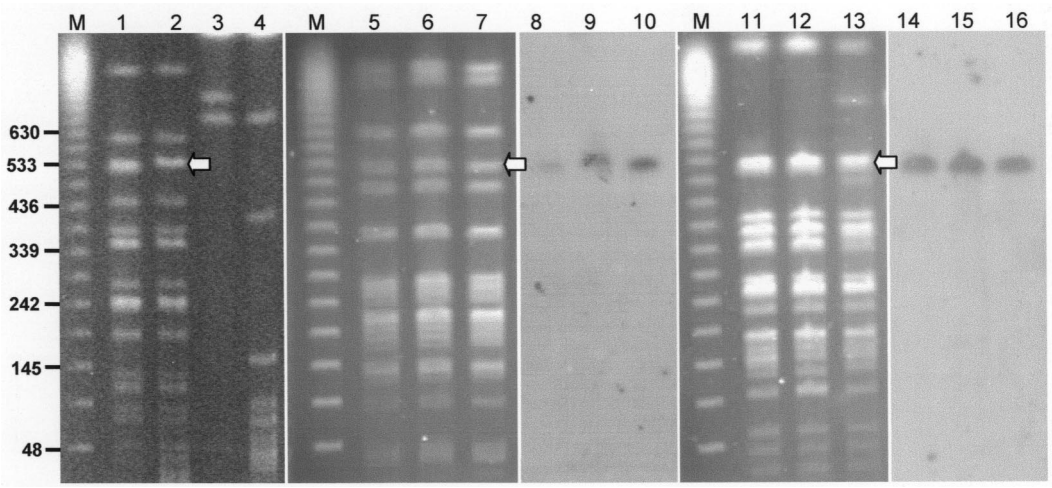

FIG. 1. PFGE mapping of the BCESM in CF strains from patients with chronic infection. PFGE macrorestriction and Southern hybridization results of *B. cenocepacia* CF strains are shown. Lanes are as follows: M, molecular size markers with relevant fragment sizes (in kilobases) indicated on the left of the figure; 1 and 2, CEP54A and CEP54B, respectively, digested with *Spe*I; 3 and 4, CEP54A and CEP54B, respectively, digested with *Swa*I; 5, 6, and 7, strain type 2 sequential isolates recovered from patient A, after 1, 2, and 3 years of chronic infection (*Spe*I digests); 8, 9, and 10, corresponding Southern blot of lanes 5 to 7 hybridized to the BCESM; 11, 12, and 13, strain type 4 isolates recovered from patient B, after 1, 3, and 5 years of infection (*Spe*I digests); 14, 15, and 16, Southern blot of lanes 11 to 13 hybridized to the BCESM. The arrow indicates the conserved 503-kb *Spe*I fragment to which the BCESM hybridized in all strains.

then introduced into *B. cenocepacia* strain K56-2 by conjugal transfer in a triparental mating with the helper plasmid pRK2013 as previously described (17). Mutants which had undergone allelic exchange were selected by plating on trimethoprim containing media and evaluated for correct double-crossover homologous recombination by PCR with the appropriate primer set (Table 2), PFGE analysis, and conventional Southern hybridization restriction fragment length polymorphism (RFLP) analysis (36).

Agar bead infection model. Groups of 15 male Sprague-Dawley rats (150 to 170 g; Charles River Canada, Inc.) were tracheostomized under anesthesia and inoculated with approximately 10^5 CFU of K56-2 or the mutant strains (Table 1) embedded in agar beads as previously described (5, 37). On days 1 and 14 postinfection (p.i.), the lungs from five animals per group were removed aseptically and homogenized (Polytron Homogenizer; Brinkmann Instruments, Westbury, N.Y.) in 3 ml of phosphate-buffered saline, serially diluted and plated on tryptic soy agar and *B. cepacia* selective agar (13), and incubated at 37°C to determine the number of bacteria present in the lung. Lung homogenates from the mutant strains were also plated on medium with trimethoprim (100 μg/ml) to confirm that the mutations were stable throughout the course of the infections. Lungs from five additional animals per group were examined for quantitative histopathological changes as previously described (39) with the following modifications. The lung sections were scanned using an Epson 1650 scanner. Areas of inflammation, characterized by cellular infiltration consisting of predominantly polymorphonuclear leukocytes and inflammatory exuviate, were identified and digitized with Scion Image software and reported as the percentage of the total area of the lung section that was covered by inflammatory exudate. To determine the ability of the amidase mutant to persist in the lungs, groups of 15 rats were each infected with approximately 5×10^5 CFU of K56-2 or K56-2*amiI*, and on days 1, 14, and 28 p.i. lungs were removed from groups of animals and quantitative bacteriology analysis was performed as described above.

RESULTS

Stability of the BCESM genomic region. Epidemiological studies had demonstrated that BCESM was not possessed by all *B. cenocepacia* strains (19, 22), suggesting it may be part of an unstable genomic region. In vitro characterization of a *B. cenocepacia* III-B CF strain, CEP54 (RAPD type 17, the Cleveland epidemic lineage [25]) indicated that instability of the marker may occur. One hundred colonies from the primary culture of strain CEP54 were analyzed by BCESM colony PCR, and 28 were found to be negative for the marker. Single positive and negative clones were purified and subcultured. PCR testing demonstrated that the BCESM remained stable in the positive derivative, CEP54B (the suffix B indicates that BCESM is present), during subsequent culture passage. PFGE analysis of CEP54B and the negative clone CEP54A (the suffix A indicates that BCESM absent) demonstrated that a complex genomic rearrangement had occurred in strain CEP54A resulting in loss of BCESM encoding DNA (Fig. 1, lanes 1 to 4).

To determine if the BCESM was stable during chronic CF infection we analyzed sequential isolates from five patients with *B. cenocepacia* infection of strain type 1, 2 (2 patients), 4, and 6, which all encode the BCESM (27, 28). The PFGE fingerprints of the strains were stable (for up to 11 years in the case of strain type 6; data not shown), with BCESM hybridizing genomic fragment remaining intact throughout chronic lung infection. PFGE and hybridization data for strain types 2 and 4, over a 3- and 5-year period of infection, respectively, are shown in Fig. 1 (lanes 5 to 16). Strain type 2 is the same type as the genome project strain *B. cenocepacia* J2315 (25), and the 503-kb *Spe*I fragment to which the BCESM hybridized (Fig. 1B) was the same as that predicted from the genome sequence data. The same isogenic 503-kb *Spe*I fragment hybridized to the BCESM in strain types 1 and 4 (Fig. 1), and our experimental strain K56-2 (also strain type 2 [25]). These observations from clinical CF infection demonstrate that the BCESM region is stable during infection and conserved on a macrorestriction scale, and they suggest that the loss of the marker observed in *B. cenocepacia* III-B strain CEP54A was rare.

Bioinformatic analysis of BCESM genomic locus. Two approaches were used to map the gene content of the DNA surrounding the original BCESM: nucleotide sequence data from the *B. cenocepacia* genome project and Southern hybridization using PCR probes designed from the sequence data

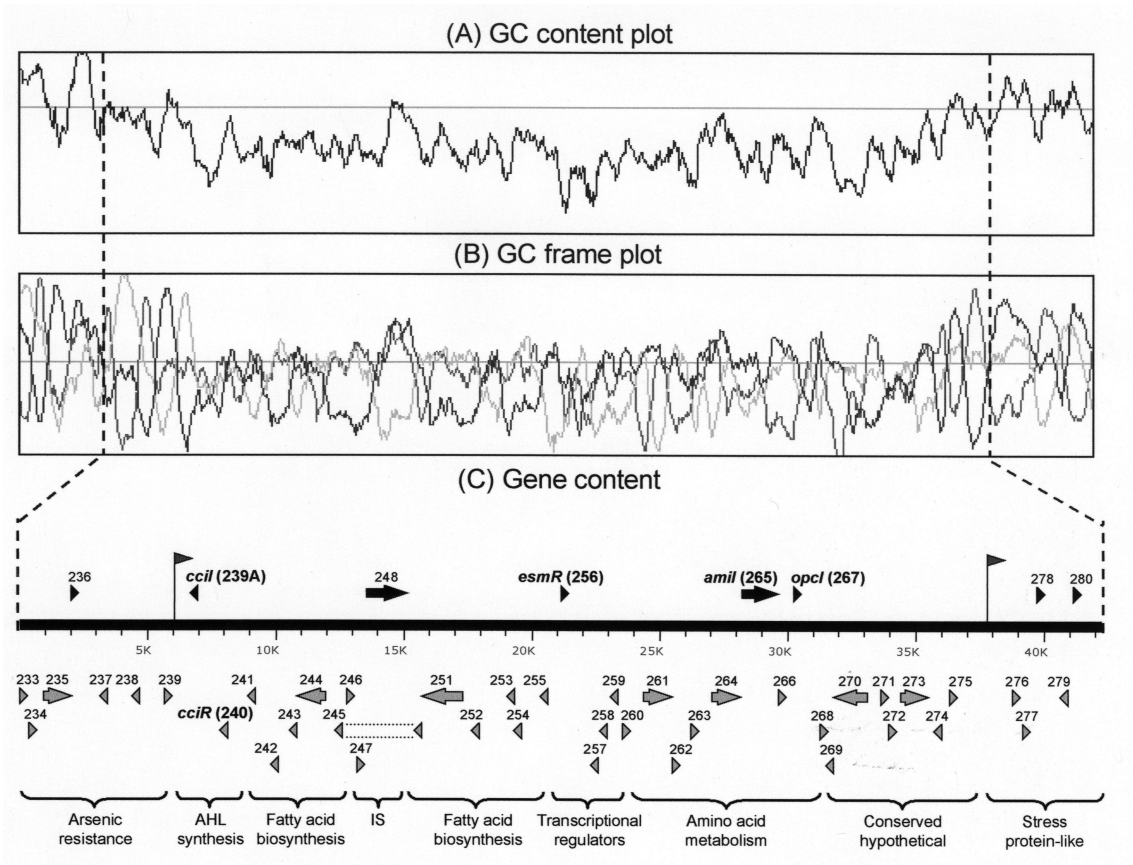

FIG. 2. Bioinformatic analysis of the *B. cenocepacia* island. (A and B) GC content plots and GC frame plots are shown, respectively; GC percentage was calculated over a window size of 500 bases, and the mean GC content of the second chromosome, 67.3%, is indicated by the line across each panel. (C) The gene content of the DNA in the BCESM region is shown, with each predicted CDS designated by its BCAM number or designated name. Genes mapped by Southern hybridization are shown as black arrows above the sequence bar (size in kilobases indicated on bar); other CDSs are indicated below the sequence bar as grey arrows. The predicted functions of each gene cluster are shown below the brackets. The position of the 13-bp direct imperfect repeats which flank the cci are shown by the arrows linked to the sequence bar.

(see below). Hybridization of BCESM probe to PFGE-separated replicons of *B. cenocepacia* strains demonstrated that the marker was encoded on the second-largest chromosome of the multireplicon genome (data not shown); this observation was corroborated by the *B. cenocepacia* genome sequence data. Bioinformatic analysis of the BCESM encoding region of *B. cenocepacia* J2315 was immediately striking, revealing that the original BCESM (27) was part of a large low-GC-content genomic island (Fig. 2A). The island was approximately 34 kb with a GC content of 61.7%, compared with the second replicon mean of 67.3% (Fig. 2A) and the overall *B. cenocepacia* genome mean of 66.9%. The reading frame position-specific GC usage was also very different from the rest of the genome (Fig. 2B) indicating that the DNA may have been recently acquired by *B. cenocepacia* from a different host organism (16). Because of this novel finding and the historical association of the BCESM with *B. cenocepacia*, the region was designated as the *B. cenocepacia* island (cci).

Preliminary annotation of 43 kb of sequence spanning the cci revealed the presence of 47 predicted CDSs (formal annotation will be completed and published by the Sanger Institute). Thirty-five CDSs were within the low-GC-content portion of the cci (BCAM0239A to BCAM0275), and 12 CDSs were outside this region (BCAM0233 to BCAM0239A and BCAM0276 to BCAM0280 [Fig. 2C]). Dot plot analysis of the genomic region indicated the presence of 13-bp imperfect direct repeats which flanked the island (Fig. 2C). The downstream repeat, 5′-TTTCTGCTCAGGC-3′, was within CDS BCAM0239, and the upstream repeat, 5′-TTTCTGCCCAGGC-3′, was within the intergenic region between BCAM0275 and BCAM0276 (the single mismatched base is underlined). The size of the cci between these repeats was 31.7 kb, smaller than initially predicted from the GC content plots (Fig. 2A and B) and the derived island possessed an even lower GC content of 60.8%. Neither repeat sequence occurred elsewhere in the *B. cenocepacia* genome and a BLAST search of the DNA databases for short nearly exact sequence matches demonstrated only one perfect prokaryotic match to the downstream repeat which was within the flagellin gene of *E. coli* serotype O128:H2 (4). However, both repeat sequences occurred widely within eukaryotic organisms.

Four functional gene clusters and one hypothetical gene set were encoded by the cci. Genes with homologies to known genes included: (i) autoinducer synthesis loci, with an *N*-acyl homoserine lactone (AHL) synthase gene and corresponding transcriptional regulator, which were designated as *cciI*

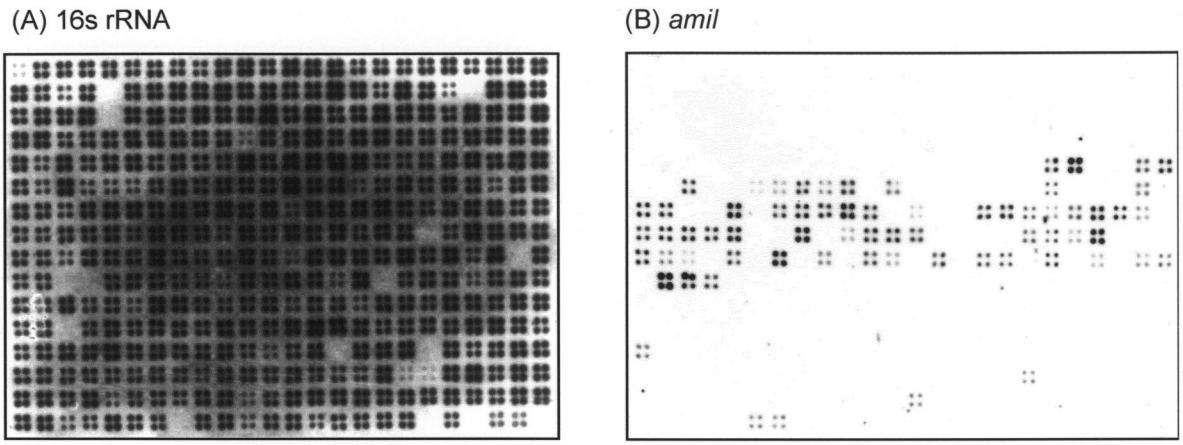

FIG. 3. Southern hybridization to BCC genomic DNA arrays. (A) Control hybridization to pooled 16S rRNA gene probes. (B) Specific hybridization of the *amiI* gene probe to strains of *B. cenocepacia* encoding the BCESM DNA.

(BCAM0239A) and *cciR* (BCAM0240), respectively (Fig. 2C), to distinguish them from the characterized CepIR autoinducer loci of *B. cenocepacia* (17); (ii) a cluster of genes with homologies to fatty acid biosynthesis genes (BCAM0241 to BCAM0246 and BCAM0251 BCAM0255) that contained a copy of an IS*66* family transposon (BCAM0247 and BCAM0248; originally described in the *Agrobacterium tumefaciens* Ti plasmid) inserted into BCAM0245, a putative decarboxylase; (iii) a set of putative transcriptional regulator loci including the previously described *esmR* gene (BCAM0256 to BCAM0259); and (iv) a cluster of genes associated with amino acid transport and metabolism (BCAM0260 to BCAM0268 [Fig. 2C]). In addition to these gene clusters with putative functions, the cci also encoded a cluster of seven CDSs with homology to other conserved or hypothetical genes with no currently known functions (BCAM0269 to BCAM0275 [Fig. 2C]). The cci was flanked by a downstream cluster of arsenic and antibiotic resistance-associated genes (BCAM0233 to BCAM0239) and upstream conserved genes with homologies to known stress proteins (BCAM0276 to BCAM0280 [Fig. 2C]).

Southern hybridization mapping of the cci. Hybridization of selected cci genes against a collection of 241 genetically distinct BCC strains representative of each genomovar/species was used to determine the extent and prevalence of the cci. PCR hybridization probes were designed from the *B. cenocepacia* J2315 genome sequence and the following genes were targeted for mapping (Table 2). BCAM0236, BCAM0278, and BCAM0279 were selected because they were outside the low GC island (Fig. 2C) and in the cases of BCAM0236 and BCAM0278, demonstrated good homology to known genes (Table 2). Within the cci, the BCESM (27), the AHL synthase, *cciI*, and the IS*66* family transposase, BCAM0248, were selected (Table 2). In addition, a putative amidase (related to pfam01425), designated *amiI*, and a predicted porin, designated *opcI*, related to the OmpC family of gram-negative porins (pfam 00267 [Table 2]) were also selected. PCR probes labeled with digoxigenin were hybridized to the genomic DNA arrays as shown in Fig. 3 and the prevalence of each selected gene is shown in Table 3.

The BCESM probe hybridized exclusively to *B. cenocepacia* strains, corroborating previous findings (25, 27). The BCESM

TABLE 3. Prevalence of cci genes mapped by Southern hybridization with the BCC genomic DNA arrays

Gene probe	No. of strains that hybridized to the indicated BCC genomovar								
	I (n = 23)	*B. multivorans* (n = 53)	*B. cenocepacia* (n = 88)	*B. stabilis* (n = 12)	*B. vietnamiensis* (n = 21)	VI (n = 2)	*B. ambifaria* (n = 19)	*B. anthina* (n = 10)	*B. pyrrocinia* (n = 13)
Arsenic resistance gene (BCAM0236)	4	13	46	3	3	0	8	0	0
AHL synthase (*cciI*)	0	0	48[a]	0	0	0	0	0	0
IS transposase (BCAM0248)	1	0	6	0	0	0	0	2	3
BCESM (*esmR*; BCAM0256)	0	0	48[a]	0	0	0	0	0	0
Amidase (*amiI*)	0	0	48[a]	0	0	0	0	0	0
Porin (*opcI*)	0	0	48[a]	0	0	0	0	0	0
Stress protein (BCAM0278)	0	0	20	1	0	0	7	0	0
Hypothetical gene (BCAM0280)	0	0	17	0	0	0	0	0	0

[a] AHL synthase, amidase, and porin gene probes hybridized to exactly the sames strains as the BCESM probe across the array.

was present in 55% (48 strains) of the *B. cenocepacia* strains represented on the genomic DNA arrays (Table 3). The *cciI*, *amiI*, and *opcI* genes which were within the cci, all demonstrated the same prevalence as the original BCESM, hybridizing exclusively to *B. cenocepacia* strains (Table 3). In addition, all the latter probes hybridized to exactly the same strains, suggesting that their distribution was linked. Hybridization of these probes to PFGE-separated DNA also demonstrated that they colocalized to the same genomic region as the BCESM in *B. cenocepacia* (data not shown). The only gene within the island which did not demonstrate an identical distribution to the BCESM was the IS*66* family transposase. It was present in a few genomovar I, *B. anthina*, and *B. pyrrocinia* strains, as well as within the *B. cenocepacia genome* strain J2315 and clonal ET12 strains (including K56-2 [25]). The downstream arsenic resistance gene probe, BCAM0236 (Table 2), hybridized to five other BCC species in addition to *B. cenocepacia* (Table 3), with only *B. cepacia* genomovar VI, *B. anthina*, and *B. pyrrocinia* not possessing homologous genes. Probes directed to the upstream flank of the cci produced a different distribution profile. The stress protein-like probe, BCAM0278, hybridized only to *B. cenocepacia* subgroup III-A strains (18 of 31 III-A strains on the array). The hypothetical gene probe, BCAM0280, detected homologs in *B. cenocepacia*, *B. stabilis*, and *B. ambifaria* (Table 3). Overall, the hybridization mapping indicated that gene probes outside the low GC cci were not closely linked in their distribution and that the *cciI*, *amiI*, and *opcI* which were always associated with an intact cci, were conserved across all *B. cenocepacia* strains possessing the original BCESM.

Mutagenesis of cci genes. To establish a functional role for the cci in infection, directed mutagenesis of the AHL synthase, amidase, and porin genes was carried out. These genes were selected because they possessed predicted nonregulatory functions, were part of a putative functional cluster of genes (Fig. 2) and were known from the hybridization data to be carried on the cci in all BCESM positive strains. In addition, the CepIR quorum-sensing system of *B. cenocepacia* had already been shown to play a critical role in virulence factor production (17), and hence the role of the additional CciIR was deserving of evaluation. Knockout mutants were created by homologous recombination with gene constructs interrupted by a trimethoprim cassette inserted at single restriction sites within each CDS as shown in Table 1. Mutants arising from double-crossover homologous recombination were screened by PCR, Southern hybridization and PFGE to confirm they possessed the correct genotype. Southern hybridization analysis of all three mutants probed with the *amiI* gene (Table 2) is shown in Fig. 4A and B. The amidase mutant, K56-2*amiI*, demonstrated the correct 1.1-kb increase in RFLP profile expected from the directed mutagenesis; the porin mutant also showed the same increase as it was encoded on the same restriction fragment as the amidase gene. Southern hybridization with the cciI and opcI probes (Table 2) also indicated that each of the latter mutants possessed the correct genotype (data not shown). Finally to confirm that no additional genomic rearrangements had occurred during the mutagenesis procedure, PFGE analysis was performed and demonstrated that all three mutants were isogenic in macrorestriction fingerprint to their parental strain, K56-2 (Fig. 4C). In vitro, the *cciI*, *amiI*, and *opcI* mutants did not demonstrate any altered growth patterns or alterations in observable phenotype when basic growth kinetics were examined. In addition, the genotype of each mutant remained stable when passaged for 10 generations without selection for trimethoprim.

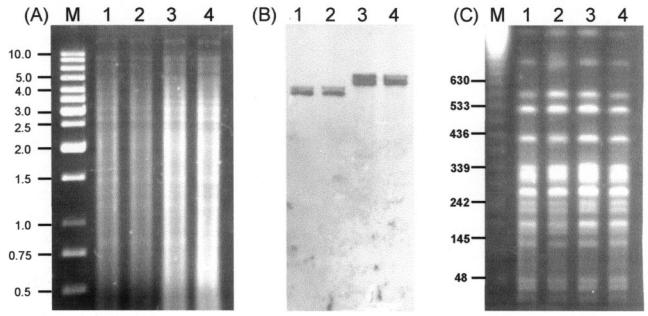

FIG. 4. Confirmation of the *B. cenocepacia* K56-2*cciI*, K56-2*amiI*, and K56-2*opcI* mutants. (A and B) Conventional *Sal*I RFLP mapping of the amidase gene mutation. (A) Lanes are as follows: M, 1-kb molecular size marker; 1, K56-2; 2, K56-2*cciI*; 3, K56-2*amiI*; and 4, K56-2*opcI*. (B) Corresponding Southern hybridization probed with the amidase gene probe (Table 2). (C) PFGE *Spe*I analysis of the following strains: lane 1, K56-2 parent; lane 2, K56-2*cciI*; lane 3, K56-2*amiI*; and lane 4, K56-2*opcI*. PFGE molecular size markers were run in lane M, and the sizes of relevant bands are indicated.

Infection studies on cci mutants. To establish if cci-encoded genes played a role during infection, the ability of the *cciI*, *amiI*, and *opcI* mutants to establish chronic respiratory infection was compared to the parent strain using the rat agar bead infection model (5, 37). Lungs were removed on day 1 p.i. to determine the ability of the strains to establish an infection. Similar numbers of bacteria were recovered from the lung on day one indicating that the mutants were able to establish an infection (Table 4). Quantitative bacteriology was also performed on day 14 p.i. Approximately 1 log fewer bacteria were recovered from lungs infected with the amidase mutant compared to lungs infected with K56-2 (Table 4). To determine if functional amidase was required for persistence in the lung, a second experiment was performed and quantitative bacteriology analysis was performed on days 1, 14, and 28 p.i. On day

TABLE 4. Ability of *B. cenocepacia* cci mutants to establish persistent respiratory infections

Strain Expt. 1	Mean log CFU/ml/per lung ± SD[a] at day:	
	1 p.i.	14 p.i.
Expt 1		
K56-2	6.1 ± 1.4	5.3 ± 0.9[b]
K56-2*cciI*	6.0 ± 1.2	4.7 ± 1.2
K56-2*amiI*	6.8 ± 0.3	4.2 ± 0.8
K56-2*opcI*	5.6 ± 0.6	5.4 ± 0.4
Expt 2		
K56-2	6.7 ± 0.2[c]	5.1 ± 0.5
K56-2*amiI*	5.6 ± 1.3[c]	4.2 ± 0.4[d]

[a] Five animals per group unless indicated otherwise.
[b] Six animals per group.
[c] Four animals per group.
[d] Significantly different from result for K56-2 ($P < 0.05$; t test for unpaired observations).

TABLE 5. Comparison of lung histopathology in animals infected with *B. cenocepacia* mutant and parent strains

Strain	% Inflammation[a] (mean ± SD)	*P*
K56-2	28.2 ± 0.2	
K56-2*cciI*	14.0 ± 5.7	<0.05
K56-2*amiI*	23.8 ± 8.3	NS[b]
K56-2*opcI*	13.6 ± 4.8	<0.05

[a] Five animals per group.
[b] NS, not significant.

14 p.i. approximately 1-log fewer bacteria were again recovered from the lungs of animals infected with the amidase mutant compared to K56-2 (Table 4; $P < 0.05$, *t* test for unpaired observations). A 1-log difference in the number of bacteria recovered was also observed on day 28 p.i. although the difference in this case was not quite statistically significant (data not shown). These data suggest that amidase does play a role in the ability of K56-2 to persist in the lung but that it is not an essential requirement for persistence.

The effect of the mutations on virulence of K56-2 was also determined by performing quantitative histopathology analysis on lungs on day 14 p.i. Mutations in the AHL synthase and porin genes resulted in significantly less histopathological changes in the lungs (Table 5). Scans of representative lung sections from each group of animals are shown in Fig. 5. The inflammation in animals infected with these strains was approximately 50% less than that observed in animals infected with K56-2, despite similar numbers of bacteria being present in the lungs (Table 4). These data indicate that the AHL synthase and porin genes contribute significantly to the virulence of *B. cenocepacia*. The amidase mutant did not demonstrate any alteration in lung histopathology when compared to the parental strain K56-2. The *amiI* gene and *opcI* are very close to one another on the island, with only CDS BCAM0266 intervening (Fig. 2). The contrasting in vivo growth (Table 4) and inflammatory (Table 5) phenotypes of the knockout mutants of each gene indicate that no polar effects were associated with directed mutagenesis of the amidase and porin loci.

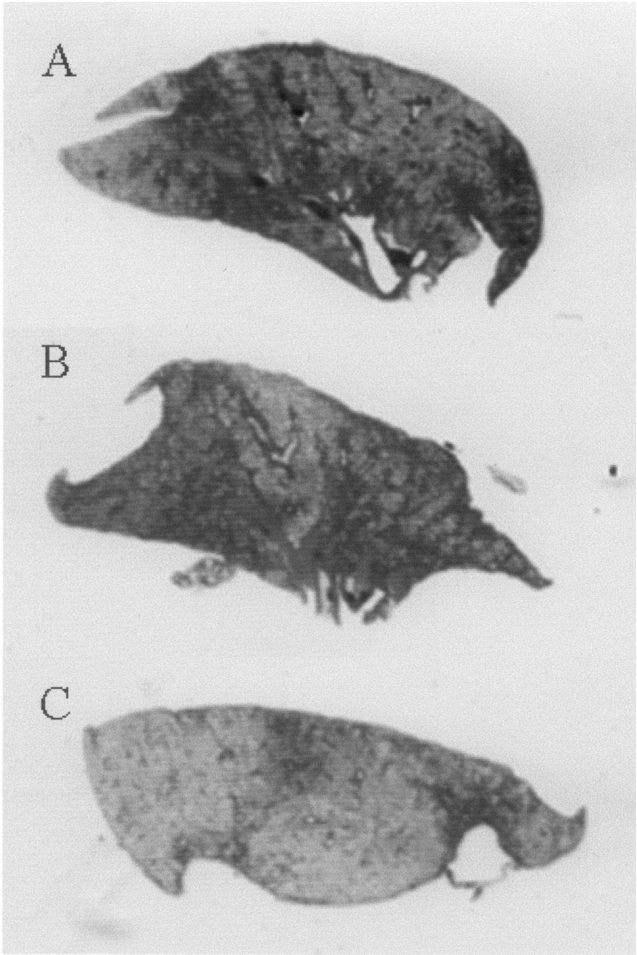

FIG. 5. Scans of representative lung sections from animals infected with K56-2 (A), K56-2*cciI* (B), or K56-2*opcI* (C). Regions of darker staining are indicative of areas of inflammation. The inflammation calculated for the sections in panels A to C are 28, 13, and 11%, respectively.

DISCUSSION

Our study has demonstrated that the BCESM is part of a novel genomic island encoding genes which play a significant role in the virulence of *B. cenocepacia*. The BCESM was originally identified by RAPD analysis (27), a strain typing method which we have found particularly useful for typing and molecular epidemiological analysis of several bacterial species (23, 24, 40). In contrast, other investigators have considered RAPD strain fingerprinting methods to lack reproducibility and to be of limited epidemiological use (43). The discovery of the BCESM by RAPD analysis (27), its use as a marker of clinical risk during *B. cenocepacia* CF infection (28, 40), and the fact that we have now shown that it forms part of a putative pathogenicity island, demonstrates the broad utility of PCR-mediated strain typing methods such as RAPD analysis. These PCR fingerprinting methods may be used for both typing and as differential genomic display methods able to identify useful strain specific markers as shown by this work.

In comparison to other known pathogenicity islands (12), the *B. cenocepacia* cci possesses most of the features of such horizontally acquired genomic regions. It has an atypical GC content and a large size, possesses flanking direct repeats, has associated insertion (IS) elements, is epidemiologically linked to virulent strains, and most importantly encodes multiple genes—at least three of which we have demonstrated to play a role during chronic lung infection. However, its functional gene content is very unusual in comparison to classical gram-negative pathogenicity islands which often encode type III secretion systems, adhesins, and toxins (12). Of the genes encoded by the cci, only those associated with quorum sensing (*cciI* and *cciR* [Fig. 2]) constitute members of genetic systems previously implicated in bacterial virulence (17, 38). As far as we are aware, although it is known that quorum-sensing genes may regulate virulence factors genes encoded on pathogenicity islands (12), the cci is the first pathogenicity island to actually encode classical LuxRI homologs. IS elements and transcriptional regulators are present on many pathogenicity islands (12) and are also present on the cci, although their role in

virulence remains undetermined. The IS*66* family IS sequence (BCAM0248 [Fig. 2]) in the cci may also be a recent IS into the element, as it was only found in *B. cenocepacia* strains of the same epidemic lineage, ET12 (15, 21, 41), as the genome strain J2315 (Table 3). The other cci gene clusters associated with fatty acid metabolism, amino acid transport, and metabolism or of a conserved hypothetical nature (Fig. 2) are unusual and not found on other pathogenicity islands. Overall, the cci may be considered to constitute a genomic island which has features of both pathogenicity and metabolic islands (12).

It was already known that the BCESM DNA was encoded by transmissible strains (27) associated with high mortality in patients with CF (28); these data clearly link the presence of the *B. cenocepacia* cci to virulent *B. cenocepacia* strains. However, the cci was also absent in significant numbers of *B. cenocepacia* strains which were all associated with CF infection. Detailed clinical outcome data from infected CF patients need to be gathered to determine if cci encoding strains possess significantly greater virulence than strains which lack the island. Our animal infection model results with cci isogenic mutants suggests that the cci does function to enhance the virulence of *B. cenocepacia*. The high-prevalence data from this and previous studies (19, 22, 27, 40) suggest that *B. cenocepacia* strains of *recA* phylogenetic subgroup III-A may have been the original strains to acquire the cci. The cci appears very stable and intact in *B. cenocepacia* III-A strains, remaining present throughout chronic lung infection and with only one case of in vitro instability detected in *B. cenocepacia* III-B strain CEP54A (Fig. 1). A recent controversy in BCC research is the distinction between environmental and clinical strains, and the use of these bacteria as agents of biological control or bioremediation (30). The cci does not provide grounds for distinction between clinical and environmental strains as it was present in four *B. cenocepacia* environmental strains, including strain M36, a U.S.-registered biocontrol strain which has been withdrawn from commercial use by the manufacturer (26). The virulence role of the cci described herein may in future enable selection of biotechnological strains lacking this pathogenic marker.

Our preliminary mutagenesis data on the *cciI*, *amiI*, and *opcI* demonstrate that these genes have functional roles during infection assisting in the definition of the cci as a pathogenicity island. The role *cciI* in virulence and inflammation corroborates previous data on quorum sensing in *B. cenocepacia* (17, 38). Mutation of the *B. cenocepacia* CepIR system, the first LuxIR homologs to be identified in this species, did not completely prevent quorum sensing activity (17), suggesting that the bacterium may possess additional signaling systems. Bioinformatic analysis of the *B. cenocepacia* J2315 genome indicates that, other than the CepIR and CciIR genes, no further LuxIR homologs are present. The difference in virulence between the cciI mutant and K56-2 was similar to that reported for a K56-2*cepI* mutant in the agar bead model (38). These data suggest that the *cciI* is part of a second cell signaling system in *B. cenocepacia* and that both quorum-sensing systems are involved in regulation of virulence factors in this species. Further studies are in progress to determine the role of *cciI* in cell signaling in *B. cenocepacia*. Complementation of the cciI mutant has been performed and restores quorum-sensing activity to wild-type levels in vitro; in addition, construction of a double *cepI cciI* mutant results in a lack of detectable AHL production in *B. cenocepacia* (R. J. Malott, A. Baldwin, E. Mahenthiralingam, and P. A. Sokol, Abstr. Am. Soc. Microbiol. N.W. Branch Meet. 2003, abstr. 77, p. 97, 2003). However, it is not known if the CciIR system works in isolation or in concert with other genetic systems on the island. The adjacent fatty acid biosynthesis genes are oriented in the same direction as the *cciI* and *cciR* genes, and could potentially be transcribed together with them. This raises the possibility that these genes may be involved in modifying in some way the lipid moieties on the AHL molecule(s) generated by this system. It is also likely that the IS element present in all ET12 strains (Table 3) would have an effect on this putative modification.

It is not unusual for bacteria to have multiple cell signaling systems. In addition to the CepIR system, *B. vietnamiensis* has a second set of LuxIR homologs designated BviIR (8, 20). The role of the *bviIR* genes in regulation of virulence factors has not yet been determined. In some species multiple sets of quorum-sensing genes have been identified, and for at least some functions, hierarchical regulation exists where one system has control over the other. The most well characterized example of this is in *Pseudomonas aeruginosa*, where the LasRI system has dominant regulatory control over the RhlRI system (10, 31). Multiple *luxIR* homolog-based quorum-sensing systems have also been described in *Yersinia pseudotuberculosis* (3) and *Rhizobium leguminosarum* (47), and one system has been shown to have a regulatory role over the other. Studies are under way to determine if there is any regulatory relationship between the *cciIR* and the *cepIR* genes in *B. cenocepacia* or if these systems operate independently in regulation of potential virulence factors.

The amidase mutant was the only cci mutant to demonstrate a reduced rate of persistence during infection (Table 4). The AmiI protein has some homology with the GatA subunit of the glutamyl-tRNA amidase (9). However, genes encoding the other two subunits of the heterotrimeric GatABC complex are not present in this locus, and *B. cenocepacia* has a full set of *gatCAB* genes on the large chromosome. It is therefore likely that AmiI is involved in modification of the substrate of the putative amino acid transport system described below. Mutation of *amiI* is not lethal and does not alter basic in vitro growth kinetics, indicating that this system is not essential. Downstream of the *amiI* gene are a cluster of genes encoding putative amino acid transport proteins with significant homology to the highly conserved ABC-ATPase transporters (14) (BCAM0260 to BCAM0265 [Fig. 2]). The presence of these genes corroborates our hypothesis that *amiI* is involved in amino acid metabolism. If AmiI is functionally a GatA homolog, then it and the transporter-like proteins downstream will require energy in the form of ATP (14, 33), a possibility that adds further intrigue to our observation that *amiI* is required for in vivo survival, where one would consider pathogens to adopt energy efficient pathways unless they perform an important function. The *opcI* gene, which is closely related to the OmpC family of outer membrane bacterial porins (COG3203 [Fig. 2]), is upstream of *amiI* and the amino acid transport proteins, suggesting that it may function as a pore for the small molecules processed by these genes. Mutagenesis of the OpcI porin demonstrated that it plays a role in mediating inflammation at the site of infection (Table 5) suggesting that protein may, like other OmpC homologs, modulate immuno-

logical expression in order to evade host defenses (1). Complementation of the amidase and porin mutants has been achieved (E. Mahenthiralingam, unpublished data); however, as with the knockout mutants no observable phenotypic changes were noted after complementation. Further characterization of the role the amidase and porin genes will require the development of simple assays to detect their expression.

Although the exact functional role of the majority of the genes within the cci during infection remains to be determined, the preliminary analysis presented here indicates that the element is a highly novel and functionally unique genomic island. The island shares many of the attributes of a classical pathogenicity island encoding virulence associated genes; however, several of the gene clusters within the island are also linked to metabolism. Its association with bacteria from BCC is not surprising since these bacteria are metabolically, ecologically, and genetically diverse, capable of growing in a number of environments from the soil to mammalian infection. All BCC bacteria possess very large multireplicon genomes (6 to 9 Mb [22, 30]) and hence have the capacity to encode multiple novel genomic loci. The *B. cenocepacia* island is the first example of a genomic island to be described in the BCC, and its role in opportunistic infection and the natural environment is worthy of further study.

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