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<th>Identification of burkholderia cenocepacia strain H111 virulence factors using nonmammalian infection hosts</th>
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<tr>
<td><strong>Author(s)</strong></td>
<td>Schwager, Stephan; Agnoli, Kirsty; Köthe, Manuela; Feldmann, Friederike; Givskov, Michael; Carlier, Aurelien; Eberl, Leo</td>
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Identification of *Burkholderia cenocepacia* Strain H111 Virulence Factors Using Nonmammalian Infection Hosts

Stephan Schwager,a Kirsty Agnoli,a Manuela Köthe,** Friederike Feldmann,** Michael Givskov,b,c Aurelien Carlier,a Leo Ebert*a

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*Burrholderia cenocepacia* H111, a strain isolated from a cystic fibrosis patient, has been shown to effectively kill the nematode Caenorhabditis elegans. We used the *C. elegans* model of infection to screen a mini-Tn5 mutant library of *B. cenocepacia* H111 for attenuated virulence. Of the approximately 5,500 *B. cenocepacia* H111 random mini-Tn5 insertion mutants that were screened, 22 showed attenuated virulence in *C. elegans*. Except for the quorum-sensing regulator cepR, none of the mutated genes coded for the biosynthesis of classical virulence factors such as extracellular proteases or siderophores. Instead, the mutants contained insertions in metabolic and regulatory genes. Mutants attenuated in virulence in the *C. elegans* infection model were also tested in the *Drosophila melanogaster* pricking model, and those also attenuated in this model were further tested in *Caenorhabditis elegans*. Six of the 22 mutants were attenuated in *D. melanogaster*, and five of these were less pathogenic in the *G. mellonella* model. We show that genes encoding enzymes of the purine, pyrimidine, and shikimate biosynthesis pathways are critical for virulence in multiple host models of infection.

The *Burkholderia cebapcia* complex (Bcc) consists of 17 closely related, but phenotypically diverse, bacterial species. Strains of the Bcc have been isolated from soil, the rhizospheres of plants, groundwater, industrial settings, hospital environments, and infected humans and animals. Some strains could be valuable for biotechnology, as they can be used for bioremediation, as biological pest control agents, and for plant growth promotion. Conversely, some strains from the Bcc can act as problematic opportunistic pathogens in patients suffering from cystic fibrosis (CF) and also in immunocompromised individuals (1–4). *B. cenocepacia* and *Burkholderia multivorans* are currently the Bcc species most frequently isolated from clinical samples (1, 4). In CF patients these species can cause serious infections, leading to rapid decline in lung function and an often fatal pneumonia (known as “cepacia syndrome”) (5, 6).

Over the past decade, substantial progress has been made in identifying and characterizing the virulence determinants and infection mechanisms of Bcc strains (7). These studies used different Bcc strains and various infection models. Since strain-to-strain variability and the infection model used have a tremendous effect on the outcome of pathogenicity assays, knowledge of the importance of individual virulence factors in different infection hosts is sparse (7, 8).

In this study, we screened a *Burkholderia cenocepacia* H111 random insertion mutant bank for attenuated virulence in *Caenorhabditis elegans*. To exclude experimental and host-specific factors, other nonmammalian animal models were used to test those mutants which showed attenuation in pathogenicity to *C. elegans*. The first was the fruit fly, *Drosophila melanogaster*, into which bacteria were injected by pricking with a needle (9, 10). Those mutants that were also attenuated in *D. melanogaster* were further tested using the greater wax moth larva *Galleria mellonella*, which allows injection of a precise dosage of bacteria (8, 11). We checked mutants for the production of AidA, which is known to play a role in pathogenicity to *C. elegans* (8, 12), as well as for other previously identified virulence factors, including production of acyl-homo-serine lactone (AHL) signal molecules, siderophores, proteases, and polysaccharides.

**MATERIALS AND METHODS**

Organisms and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise stated, bacteria were grown at 37°C in modified Luria-Bertani (LB) broth (13) containing 5 g NaCl liter−1. Solid media contained agar to a final concentration of 1.5%. Antibiotics were added as required at final concentrations of 20 μg ml−1 gentamicin, 50 μg ml−1 kanamycin, 10 μg ml−1 tetracycline, 50 μg ml−1 streptomycin, 25 μg ml−1 spectinomycin, and 10 μg ml−1 chloramphenicol. Growth of liquid cultures was monitored by measurement of optical density at 600 nm (OD600) using an Ultraspec 3100 pro (Amersham Bioscience). Supplements were added to media to the following concentrations: 1-histidine, 1-cysteine, di-tryptophan, and adenosine, 500 μM; guanine, adenine, adenosine, and inosine, 20 μg liter−1. Killing assays were performed using *Caenorhabditis elegans* strain Bristol N2, which was obtained from the Caenorhabditis Genetics Center (University of Minnesota, St. Paul, MN). Nematodes were maintained on NG agar (14) at 20°C with *Escherichia coli* strain OP50 as a food source (15). For the *D. melanogaster* pricking assay, the *D. melanogaster* Canton-S wild-type strain was used. The flies were kept at room temperature and fed with standard sucrose cornmeal (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/harvardfood.htm). *G. mellonella* was bought from Fischerei Brumm.
Zurich, Switzerland, and kept in an incubator at 14°C. The larvae were used within 3 weeks of purchase.

**Transposon insertion mutagenesis.** The hybrid transposon (Table 1) was randomly inserted into the genome of *B. cenocepacia* H111 by triparental mating as described previously (24). Transconjugants were selected on LB medium containing kanamycin and tetracycline. These random insertion mutants were picked and grown in 150 μL LB medium in polypropylene microtiter plates (Nunc). For storage, 75 μL of 50% (vol/vol) glycerol was added and the plates were frozen at −80°C.

**DNA manipulations and sequence analysis of Tn5 mutants.** Cloning, restriction enzyme analysis, and transformation of *E. coli* were performed essentially as described previously (16). PCR was performed using TaKaRa rTaq DNA polymerase (TaKaRa Shuzo), as per the manufacturer’s instructions. Plasmid DNA was isolated with the QIAprep Spin Miniprep kit (Qiagen), and chromosomal DNA from *B. cenocepacia* was purified with the DNeasy tissue kit (Qiagen). DNA fragments were purified from agarose gels using the QIAquick gel extraction kit (Qiagen).

To ensure that the attenuated mutants bore only one transposon,
Southern blotting was employed. Genomic DNA was digested with SpII. The fragments were electrophoresed through a 1% agarose gel and transferred using a blot apparatus (Stratagene, Heidelberg, Germany) to a positively charged nylon membrane (Hybond-N+; Amersham, Cleveland, OH). The transferred DNA was UV fixed, and the blots were hybridized with digoxigenin (DIG)-coupled probe. The probe was prepared and detected using DIG High-Prime DNA labeling and detection starter kit I (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Probes for the detection of the transposons were constructed by PCR amplification of the Tn5 kanamycin cassette using Kan res-v and Kan res-r primers (17) (Table 2).

The insertion position of the transposon was determined by two different techniques, SpII cloning and arbitrary PCR. DNA sequences flanking transposon insertions were determined by arbitrary PCR as described previously (18), with some modification. Briefly, we performed two rounds of PCR amplification using a degenerate arbitrary primer to anneal to the chromosome and one specific to the mini-Tn5 transposon. Primers used in the first round were ARB6 and luxCext2 (Table 2). First-round reaction conditions were as follows: (i) 5 min at 95°C; (ii) 6 cycles of 30 s at 95°C, 30 s at 30°C, and 1 min at 72°C; (iii) 30 cycles of 30 s at 95°C, 30 s at 45°C, and 1 min at 72°C; and (iv) 5 min at 72°C. The second round of PCR amplification used 5 μl purified first-round PCR product as the template and primers ARB2 and luxCint2 (Table 2). Second-round reaction conditions were as follows: (i) 30 cycles of 30 s at 95°C, 30 s at 45°C, and 1 min at 72°C, and (ii) 5 min at 72°C. The PCR products were purified from an agarose gel and ligated into the vector pCR 2.1-TOPO.

Sequencing reactions were carried out to determine the transposon integration sites using the Seq_O primer (Table 2), which binds to the O end of the mini-Tn5 transposon. Integration sites were determined using the online BLAST search engine (http://www.ncbi.nlm.nih.gov/).

For the SpII cloning, genomic DNA was digested with SpII. This cleaved the i end of the transposon, including the kanamycin resistance gene. The digested DNA was cloned into pUC19 and transferred into E. coli DH5α. Bacteria containing a transposon fragment were selected on LB plates containing kanamycin. The sequence of the inserted DNA was found by sequencing with standard M13 primers, Kan res-v and Kan res-r (Table 2).

Complementation of the Tn5 mutants using B. cenocepacia H111 cosmids library. Chromosomal DNA was extracted from B. cenocepacia H111 using a standard protocol (19). The DNA was partially digested with EcoRI and ligated into cosmids pRG930 (20). Packaging and of the cosmids into E. coli HB101 cells and titer determination were carried out using the Gigapack III Gold packaging reaction kit from Invitrogen as described in the instruction manual but with the omission of the dilution step of the titer determination reaction. The whole cosmids library was pooled and introduced into each transposon mutant by triparental mating (21). Complemented mutants were selected on ABC minimal plates containing streptomycin and spectinomycin. Cosmids were then extracted using a commercial C8 HSLs were used as a standard. The cells were incubated in FluoroNunc Polysorb microtiter plates (Nunc Roskilde) for 6 h in the dark at 30°C. Fluorescence was measured with a Lumex Fluoro 320 Plus reader (Bio-Tek Instruments, Winooski, VT), and the relative fluorescence units (RFU) were determined.

**TABLE 2 Primers used in this study**

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**G. mellonella killing assays.** The G. mellonella infection assay was performed as described previously (8). Modifications were made to the protocol as follows. To prevent contamination, the Tn5 mutants’ growth medium was supplemented with kanamycin. For hemolymph extraction, larvae were first sterilized by brief immersion in absolute ethanol. Hemolymph was removed using a syringe with a 22-gauge needle. Approximately 100 μl was obtained per larva. Dilutions were plated for bacterial enumeration. Caterpillars in the final larval stage were purchased from Fischerei Brumann, Zurich, Switzerland.

**D. melanogaster pricking assays.** D. melanogaster Canton-S wild-type flies were used for the pricking procedure. The flies were bred on standard cornmeal sucrose medium and kept at room temperature. The assay was performed as described previously (10). Modifications were made to this protocol as follows. Two- to 9-day-old male and female flies were used in this experiment. For anesthetization, ether was used instead of a CO₂ pad. For each trial, duplicate sets of 15 flies were used and the experiment was performed in triplicate. The negative-control flies were inoculated with a 10 mM MgSO₄ solution. If more than 5 flies died due to pricking injury, the experiment was repeated. The infected flies were incubated at 26°C.

**AHL quantification.** Strains were grown to an OD₆₀₀ of 3.0. Bacteria were collected by centrifugation at 6,500 rpm for 5 min and the supernatant fluid sterilized. One hundred microliters of supernatant was mixed with 100 μl exponential-growth-phase Pseudomonas putida F117 (PAS-C8-Gm³). This strain is highly specific for C₈ homoserine lactone (HSL) detection. Commercial C₈ HSLs were used as a standard. The cells were incubated in FluoroNunc Polysorb microtiter plates (Nunc Roskilde) for 6 h in the dark at 30°C. Fluorescence was measured with a Lumex Fluoro 320 Plus reader (Bio-Tek Instruments, Winooski, VT), and the relative fluorescence units (RFU) were determined.

**Determination of siderophore production and extraction of pyochelin.** Siderophore activity of the H111 mutants was tested on CAS agar as described previously (25). CAS plates were inoculated with 5 μl culture at an OD₆₀₀ of 1.0, allowed to dry, and incubated at 37°C for ~48 h.

**Pathogenicity screen with C. elegans.** Screening was carried out on NG agar plates as described previously (22, 23). H111 mutants were grown at 37°C overnight in LB broth in microtiter dishes (Nunc). Fifty microliters of the respective cultures was spread on NG agar plates in 24-well multilpates (Greiner, Germany). Plates containing bacteria were incubated overnight at 37°C. After a brief incubation at room temperature to allow cooling, plates were inoculated with 20 to 30 synchronized L4 worms of the C. elegans Bristol N2 wild-type strain (obtained from the Caenorhabditis Genetics Center, University of Minnesota, Minneapolis, MN) (24). The plates were then incubated at 20°C, and live worms were counted after 2 days. In the initial screen approximately 230 24-well plates were tested in a single replicate. All mutants identified in the initial screen were retested in five independent replicates.
Siderophore activity was determined by comparison of halo diameter (a larger halo indicates higher siderophore production) and color (25, 26).

For pyochelin extraction, mutants were grown overnight in 200 ml NG medium to reflect the growth conditions used for the initial identification of mutants attenuated in virulence to C. elegans. This ensured both the growth of the mutants and the relevance of the results to pathogenicity in the C. elegans model. Bacteria were collected by centrifugation, and the pH of the spent culture supernatants was adjusted to ~2.0 by the addition of HCl. The siderophores were extracted twice by addition of 0.4 volume dichloromethane. The organic phase was removed by rotary evaporation, and the residue was resuspended in 200 to 250 µl methanol.

The extracts were analyzed by thin-layer chromatography on silica 60 plates (VWR) with chloroform-acetic acid-ethanol at 90:5:2.5 (vol/vol) as the solvent (27). Plates were dried after development and siderophores detected by UV light. Pyochelin was identified by the presence of two yellow-green fluorescent bands corresponding to the two pyochelin stereoisomers, pyochelins I and II (Rf, 0.35 and 0.37, respectively), and salicylate was visualized as a blue fluorescent band (Rf, 0.74) (28, 29).

**Determination of protease activity and EPS production.** Protease activity was determined on skimmed milk agar as described previously (30). Five microliters of overnight culture from each strain to be tested (diluted to an OD600 of 1) was dropped onto the plate and allowed to dry. The plate was incubated at 37°C for 48 h. Extracellular polysaccharides (EPS) production was determined on YEM agar supplemented with Congo red (31). EPS-positive strains showed a slimy, slightly red colony morphology after overnight incubation at 37°C, whereas EPS-negative strains turned red as a result of integration of the dye.

**Production of AidA.** Detection of AidA was performed by Western blotting. Whole-cell proteins were separated on a 15% SDS-polyacrylamide gel and then transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Eschborn, Germany). Anti-AidA antibodies (12) were used to probe the membrane. Detection reactions were performed with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Sigma, Steinheim, Germany) according to the recommendations of the manufacturer (Roche, Mannheim, Germany).

**Growth on minimal media.** The growth of the transposon mutants was tested using ABC or ABG minimal medium (32). For growth tests, 5 ml of ABC or ABG solution was inoculated with bacteria to an OD600 of 0.01 and incubated at 37°C with shaking. For the functional complementation (supplementation) of the purine mutants, 20 mg liter−1 adenine, inosine, or guanine was added to ABC minimal medium.

**RESULTS**

**Identification of B. cenocepacia H111 mutants attenuated in C. elegans and their phenotypic characterization.** It has previously been shown that on NG medium the CF isolate B. cenocepacia H111 kills C. elegans N2 within 3 days. We employed this “slow-killing” assay to screen a collection of approximately 5,500 random mini-Tn5 insertion mutants of B. cenocepacia H111 for attenuated virulence. In total, 23 mutants that reproducibly showed reduced killing after 48 h compared with the wild type were isolated (Fig. 1; Table 3). Previous work has identified several factors that contribute to the pathogenicity of B. cenocepacia (7), and we therefore tested the mutants for the production of some of these factors, including siderophores, extracellular polysaccharides (EPS), proteases, AHL quorum-sensing (QS) signaling molecules, and AidA, a protein required for nematode pathogenicity (Table 3).

**B. cenocepacia H111** produces the two siderophores ornibactin and pyochelin, which were previously shown to be important for virulence in mammals, C. elegans, and larvae of the greater wax moth G. mellonella but not alfalfa (8, 33, 34). Some of the mutants showed reduced siderophore production (Table 3). In addition, we observed that some of the mutants formed a yellow rather than a pink halo on CAS agar (Fig. 2A), which is indicative of a loss of pyochelin production (35). Extraction of culture supernatant and
TABLE 3 B. cenocepacia H111 mutants attenuated in C. elegans

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gene disrupted</th>
<th>H111 gene ID</th>
<th>Mean (SD) % dead C. elegans larvae after 48 h&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean (SD) % D. melanogaster survival time</th>
<th>Mean (SD) % surviving G. mellonella after 48 h</th>
<th>AHL production&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Protease activity&lt;sup&gt;b&lt;/sup&gt;</th>
<th>EPS production&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Siderophore production&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Expression of AidA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Growth on ABC&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>E5</td>
<td>rsaA</td>
<td>358074812</td>
<td>29 (9) &lt;72</td>
<td>NT</td>
<td>&lt;</td>
<td>&lt;</td>
<td>−</td>
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<tr>
<td>G3</td>
<td>lon</td>
<td>358073031</td>
<td>46 (10) &lt;72</td>
<td>NT</td>
<td>−</td>
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<tr>
<td>E12-2</td>
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<td>358072205</td>
<td>17 (8) &lt;72</td>
<td>NT</td>
<td>+</td>
<td>+</td>
<td>&lt;</td>
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<td>−</td>
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<tr>
<td>D6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>pyrD</td>
<td>358074754</td>
<td>23 (7) &lt;72</td>
<td>87 (15)</td>
<td>&lt;</td>
<td>&lt;</td>
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<td>+</td>
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<tr>
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<td>358072206</td>
<td>7 (5) &lt;72</td>
<td>0 (0)</td>
<td>−</td>
<td>−</td>
<td>&lt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
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</tbody>
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<sup>a</sup> −, absent.
<sup>b</sup> Halo on CAS agar of normal size but pink color absent.
<sup>c</sup> NT, not tested.

B. cenocepacia Virulence Factors

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analysis by thin-layer chromatography confirmed that these mutants produced no or reduced amounts of pyochelin (Fig. 2B).

The production of extracellular polysaccharides (EPS) is considered to enhance persistence of Bcc species in the lung, similar to alginate in Pseudomonas aeruginosa (36, 37). We tested the attenuated mutants for EPS production on YEM agar, which stimulates the production of the major Bcc polysaccharide cepacian (38, 39). Many of the attenuated mutants showed reduced EPS production (Table 3). However, a defined cepacian-deficient mutant of B. cenocepacia K56-2 was found to be as pathogenic as the wild type in C. elegans and in G. mellonella (data not shown), suggesting that the lack of EPS production is not responsible for the reduced virulence of our mutants. It should be noted that cepacian production has been shown to be important for pathogenicity in murine infection models (40, 41). Furthermore, our phenotypic characterization revealed that several of the mutants produced no or reduced amounts of AHL signal molecules, proteases, or AidA (Table 3).

Mutant D6 grew slower in LB medium, whereas the growth rates of the other mutants were indistinguishable from that of the wild type (see Fig. S1 in the supplemental material). In contrast to the wild type, however, many of the mutants showed poor or no growth on minimal medium supplemented with either glucose or citrate as a carbon source and thus were auxotrophic mutants.

Locations of transposon insertions within the attenuated mutants. The locations of the transposon insertions were determined by sequencing the DNA regions flanking the transposon (Table 3). To our surprise, only one of the disrupted genes coded for a previously described virulence factor (the QS regulator CepR), while 19 of the mutations were in metabolic genes, three mutations were in regulatory genes, and three mutants were found to have lost an entire replicon (R12, R33, and R40). The latter three mutants have been described elsewhere (42). Four of the genes identified in the transposon screen (cepR, lon, rsaA, and rsaM) code for regulatory proteins. The isolation of a cepR mutant is in full agreement with previous work that has demonstrated that the QS regulator CepR is essential for virulence in multiple infection hosts (8). One mutant was found to carry the transposon insertion in the rsaA gene. An rsaA mutant was previously identified in a screen for biofilm-defective mutants, and this gene (previously known as yclL) has been characterized as a higher-level quorum-sensing (QS) regulator (43). The transposon insertion site of another mutant that was affected in QS was mapped to the intergenic region between the divergent rsaM and cepR genes, such that expression of both genes is affected (data not shown). Finally, the lon gene codes for an ATP-dependent protease which belongs to the AAA<sup>+</sup> (ATPases associated with a variety of cellular activities) superfamily of enzymes (44). This is a widespread family of enzymes (45), responsible for diverse functions, including protein unfolding, DNA replication, and recombination (44). Disruption of the lon gene has been shown to reduce the pathogenicity of Salmonella enterica (46), Pseudomonas syringae (47), Campylobacter jejuni (48), Agrobacterium tumefaciens (49), and Brucella abortus (50). This appears to be due to the
role of Lon in the upregulation of type three secretion systems (44, 51, 52).

The 19 mutants defective in metabolic functions had mutations that fell within three major pathways: the purine biosynthetic pathway, the shikimate pathway, and the pyrimidine biosynthetic pathway. Four of the attenuated mutants identified during the C. elegans screen carried insertions within genes of the purine biosynthesis pathway (purA, purF, purL, and purD) (Fig. 3), suggesting that de novo purine biosynthesis plays an important role in the nematode pathogenicity of B. cenocepacia H111. The purF, purD, and purL genes are all positioned in the initial part of the purine biosynthetic pathway (Fig. 3), and upon inactivation of any of them, one would expect complete abrogation of purine production. The purA gene, however, is positioned later in the pathway and would not be necessary for the production of guanine- and xanthine-related purines. IMP is the first molecule containing the purine double-ring system to be formed in the purine pathway. This molecule can be used in the production of both adenine and guanine by the cell (53, 54) (Fig. 3). Growth of the purD, purF, and purL mutants could be successfully restored by the addition of inosine to ABC minimal medium (Fig. 4C, D, and E). Supplementation with adenine, but not with guanine, re-

FIG 2 Siderophore production of attenuated B. cenocepacia H111 Tn5 mutants. (A) Mutants were inoculated on CAS plates and incubated at 37°C for 48 h. The halo diameter corresponds to siderophore activity. A pink-tinted halo is indicative of pyochelin production. (B) Production of pyochelin as determined by thin-layer chromatography. Bands corresponding to pyochelins 1 and 2 (P1/P2) and salicylate (SA) are indicated. Extracts were as follows: SA, salicylate standard; wt, wild-type H111; cysl, H111 cysl; arok, H111 arok; cepR, H111 cepR.

FIG 3 The B. cenocepacia purine biosynthetic pathway. Mutants with attenuated virulence isolated in this study are circled.
stored growth to these mutants in ABC minimal medium (Fig. 4C, D, and E). Adenine can be converted to guanine via the purine metabolic pathway, but the reciprocal conversion is not possible via this pathway. The addition of inosine, adenine, and guanine to ABC medium did not affect the growth of wild-type H111 (Fig. 4A).

The pyrD and aroK mutants showed very similar phenotypic characteristics (Table 3). The pyrD gene codes for a class 2 dihydroorotate dehydrogenase, which is a key component in pyrimidine metabolism. The aroK gene encodes a component of the shikimate pathway, through which chorismate, an important precursor in the production of aromatic amino acids, is produced. Another mutant was disrupted in ilvC, a ketol-acid reductoisomerase, which plays a role in valine, leucine, and coenzyme A biosynthesis.

Of the remaining metabolic mutants, 10 had defects in amino acid biosynthesis. Four cysteine pathway mutants were all disrupted in the cysF gene (which codes for the beta subunit of a sulfide reductase), and one mutant bore an insertion in the cysB gene (a potential transcriptional regulator of the cys regulon). Two additional genes that play a role in the histidine metabolic pathway, hisG (encoding an ATP-phosphoribosyltransferase), and hisH (encoding a glutamine-amidotransferase), were found to be important for pathogenicity in the C. elegans model, as were the tryptophan biosynthetic pathway genes trpA, trpB, and trpF [encoding tryptophan-synthase alpha and beta chains and N-(5'-phosphoribosyl)anthranilate-isomerase, respectively].

The final two metabolic mutants bore insertions in genes not directly involved in amino acid biosynthesis; the ahyY (S-adenosylhomocysteine hydrolase) gene, the product of which acts as a coenzyme in cysteine and methionine metabolism, and the gatA (glutamyl-tRNA amidotransferase subunit A) gene, which plays a role in protein synthesis.

Five of the mutants attenuated in C. elegans are also less virulent in D. melanogaster and G. mellonella. Previous work has shown that some virulence factors are host specific, while other factors are important for pathogenicity in multiple infection models (8). We tested the 23 mutants showing attenuation in the C. elegans pathogenicity assay using the D. melanogaster infection model to discern host-specific and general factors. The fruit fly D. melanogaster has been shown to be a useful nonmammalian infection host for the determination of the pathogenicities of different Bcc strains (9). Of the 23 mutants with reduced C. elegans virulence, six were also strongly attenuated in the D. melanogaster infection model (Fig. 5). These six mutants were further tested for virulence using larvae of the greater wax moth G. mellonella as a host in order to validate their function as general virulence factors (Fig. 6). Five of the six mutants were attenuated compared to the wild type in this model, namely, the purD, purF, and purL purine biosynthesis mutants, the pyrD pyrimidine biosynthesis mutant, and the aroK mutant, which is defective in aromatic amino acid biosynthesis. The ilvC mutant showed attenuation in C. elegans and D. melanogaster but not in G. mellonella. It is therefore tempting to speculate that in G. mellonella but not in the other infection hosts tested, sufficient amounts of certain metabolites are available to rescue the defects of the ilvC mutant. In contrast to the purD, purF, and purL mutants, the purA mutant exhibited wild-type pathogenicity in both the D. melanogaster and the G. mellonella infection models (Table 3). In silico analysis of the B. cenoce pacia H111 genome revealed a homologous gene product (GeneID 358069869) with 51.5% identity to H111 PurA. It is possible that this gene is functionally similar to purA and is able to at least partially rescue the production of adenine-related purines in D. melanogaster and G. mellonella. The quorum-sensing regulator CepR was previously shown to be important for pathogenicity to C. elegans but not to G. mellonella (8), and our results show that
this regulator is also not essential for pathogenicity to *D. melanogaster* (Table 3).

In summary, five mutants were identified which were attenuated in all three infection hosts used. The genes inactivated in these strains (*aroK, pyrD, purD, purF*, and *purL*) may therefore encode factors that are universally important for virulence, whereas the genes inactivated in the other mutants were specific virulence factors for *C. elegans* and/or *D. melanogaster*.

**FIG 5** Virulence of wild-type *B. cenocepacia* H111 and attenuated auxotrophic Tn5 mutants in the *D. melanogaster* infection model. Fifteen flies were inoculated with bacterial culture per experiment and incubated at 26°C. Live flies were counted every 24 h postinfection. Data are based on three independent experiments and were analyzed using survival curves generated by the Kaplan-Meier statistical method. The significance of the difference between results for the wild type and the mutants was determined using the log rank (Mantel-Cox) test. For all the tested strains the *P* value was <0.0001.

**FIG 6** Virulence of wild-type *B. cenocepacia* H111 type, attenuated auxotrophic Tn5 mutants, and complemented mutant strains in the *G. mellonella* infection model. *G. mellonella* larvae were infected with approximately $2 \times 10^7$ bacteria and incubated at 30°C in the dark. Live and dead larvae were counted after 20, 24, 40, 48, and 72 h postinfection. The curves were calculated using three independent replicates. The significance of the difference between results for the wild type and the Tn5 mutants was determined using the log rank (Mantel-Cox) test and is indicated as follows: *, 0.01 ≤ *P* ≤ 0.05; **, 0.001 ≤ *P* ≤ 0.01; ***, *P* < 0.001.
Complementation and supplementation of the Tn5 metabolic mutants. The aroK, pyrD, purD, purF, and purL mutants, which showed attenuation in all the animal models tested, were complemented using a B. cenocepacia H111 cosmid library. All complemented mutants were able to grow at the wild-type rate on minimal medium with citrate as a carbon source (see Fig. S2 in the supplemental material). Furthermore, pathogenicity of the complemented mutants was at least partially restored (Fig. 6). Supplementation of nematode growth medium with histidine, cysteine, or adenosine as appropriate restored virulence to wild-type levels in the C. elegans model in all metabolic mutants except the purD mutant, which showed an intermediate level of virulence (Fig. 1; Table 3).

DISCUSSION

Over the past few years, nonmammalian infection models have been established as attractive alternatives to traditional animal models because of their practical advantages, particularly the possibility of performing high-throughput screens. In this study, we used the nematode C. elegans as an infection host to screen a B. cenocepacia H111 mutant library for attenuated strains.

Previous work has shown that the CepIR QS system is crucial for B. cenocepacia virulence in C. elegans, as it controls the expression of factors that contribute to pathogenicity in this infection model (8, 22). The finding that in one of the attenuated mutants the transposon had inactivated cepR and in two other mutants (the rsuA and lon mutants) it had affected the QS circuitry therefore validates the screening strategy. One of the QS-regulated virulence factors that has been shown to be important for killing of C. elegans is the protein AidA, although its mode of action remains to be elucidated (12). Interestingly, AidA has not been found to play a role in any other infection host tested so far, and it thus appears to be a specific virulence factor required for infection of nematodes (8). The finding that the trpA and trpB mutants produce no or greatly reduced amounts of AidA may contribute to the specific attenuation of these strains in the C. elegans model (Table 3). Supplementing the medium with tryptophan restored AidA production as well as virulence of the two mutants (Fig. 1 and data not shown), indicating that the attenuation of the two mutants is not a consequence of a secondary mutation. However, at present it is unclear how trpA and trpB, which are required for tryptophan biosynthesis, affect expression of AidA.

Other QS-regulated virulence factors of B. cenocepacia include the ZmpA and ZmpB proteases and the siderophore pyochelin (8). Proteolytic activity was shown to be important for pathogenicity in mammals but not in invertebrates or alfalfa, likely because they specifically modulate the host immune response of mammals by degrading specific tissue components such as collagen and fibronectin and by obstructing immune proteins (55–57). Hence, the abolished or lowered proteolytic activity observed for several of the mutants (Table 3) does not account for their lowered virulence in the infection models used. However, one would expect that these mutants would be attenuated in mammals. Pyochelin production in B. cenocepacia has been shown to be dependent on the availability of its precursors, salicylic acid and cysteine (58). As expected, all five mutants with insertions in the genes of the cysteine biosynthetic pathway showed a reduction in pyochelin production (Fig. 2) and an increase in the accumulation of its precursor salicylic acid. Likewise, the aroK mutant, which is defective in the biosynthesis of aromatic metabolites, including the pyochelin precursor salicylic acid, did not produce pyochelin.

Our mutant screen identified a few genes that were essential for virulence in all three nonmammalian infection models used in this study. Rather than coding for typical virulence factors, these genes were purF, purD, and purL from the purine biosynthesis pathway, pyrD from the pyrimidine synthesis pathway, and aroK from the shikimate pathway. Given that B. cenocepacia produces a battery of virulence factors (7), the inactivation of just one of these may not greatly affect the overall pathogenicity of the organism, explaining why such factors were not identified in our screen. The genes identified encode enzymes that are critical for essential anabolic pathways, and consequently these mutants were unable to grow in minimal medium. Importantly, auxotrophy per se is not the reason for the reduced virulence of these strains, as we determined that approximately 8% of the mutants in the transposon insertion library were unable to grow in minimal medium. This suggests that it is the lack or shortage of specific metabolites in each infection host that causes attenuation. In agreement with this hypothesis, we observed that nematode pathogenicity of the mutants could be restored to wild-type levels by supplementing the medium with appropriate metabolites (Fig. 1). It is important to note, however, that on NG medium, which was used for the C. elegans assays, the mutants showed no growth defects, suggesting that the reduced virulence is not a just a consequence of a lowered infection dose. In the case of the aroK mutant, we tested whether the strain could persist within infected G. mellonella larvae. At 8 days postinfection the animals did not show any disease symptoms, yet we were able to isolate the mutant from the hemolymph of the larvae (approximately 500 bacteria per μl), demonstrating that the bacteria were capable of in vivo survival. We hypothesize that the nutritional environment in the infection host supports persistence of the mutant but neither significant growth nor energy-consuming virulence factor production.

Although it remains to be determined whether our B. cenocepacia mutants are also attenuated in a mammalian infection host, it is notable that the same metabolic pathways have been identified as key systems in virulence in murine models. De novo purine biosynthesis has been shown to be essential for the virulence of a variety of pathogens, including Francisella tularensis (59), Salmonella enterica serovar Typhimurium (60), Staphylococcus aureus (61), Streptococcus pneumoniae (62), Yersinia pestis (63), Vibrio vulnificus (64), Bacillus anthracis (54, 65), Brucella melitensis (66), and Brucella abortus (67). Previous work has also shown that in V. vulnificus, pyrimidine biosynthetic genes are preferentially expressed during infection, and a pyrH mutant was attenuated in virulence (64, 68). In Listeria monocytogenes, both purine and pyrimidine biosynthetic genes were found to be upregulated in infected mammalian cells (69). In a recent study, it was demonstrated that de novo nucleotide biosynthesis is critical for survival and growth of bacteria in human serum, and therefore the purine and pyrimidine biosynthetic pathways are essential for proliferation of bacterial pathogens in the bloodstream (70). Finally, the shikimate pathway, of which the AroK protein is a part, has been demonstrated to be required for virulence of S. enterica serovar Typhimurium (71), P. aeruginosa (72), and L. monocytogenes (73). Most interestingly in the context of this study is the finding that inactivation of aroB, which is required for shikimate biosynthesis in Burkholderia pseudomallei, the causative agent of melioidosis, renders the organism avirulent. Moreover, murine challenge
studies revealed partial protection in BALB/c mice vaccinated with an aroB mutant (74). Given that the shikimate pathway is crucial to bacteria but missing in mammals (75), the enzymes involved in this pathway have been considered particularly interesting drug targets for developing nontoxic antimicrobial agents (76).

In summary, we have identified several genes in B. cenocepacia that are critical for pathogenicity in multiple nonmammalian infection hosts. These results not only further our understanding of virulence mechanisms used by this opportunistic pathogen but also have led to the identification of some potential targets for the development of novel antibacterial drugs. Some of the strongly attenuated mutants identified in this study could also be of interest for the development of live vaccines.

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