Identification of *Burkholderia cenocepacia* Strain H111 Virulence Factors Using Nonmammalian Infection Hosts

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

Department of Microbiology, Institute of Plant Biology, University of Zurich, Zurich, Switzerland[a]; Department of International Health, Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark[b]; Singapore Centre on Environmental Life Sciences Engineering, Nanyang Technological University, Singapore, Republic of Singapore[c]

Burkholderia 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-Tn*5* mutant library of *B. cenocepacia* H111 for attenuated virulence. Of the approximately 5,500 *B. cenocepacia* H111 random mini-Tn*5* 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 *Galleria mellonella*. 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 cepacia* 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} spectinomycin, 25 μg ml^{-1} streptomycin, and 10 μg ml^{-1} chloramphenicol. Growth of liquid cultures was monitored by measurement of optical density at 600 nm (OD_{600}) using an Ultraspec 3100 pro (Amersham Bioscience). Supplements were added to media to the following concentrations: L-histidine, L-cysteine, DL-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 Brumann,

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or source
E. coli		
OP 50	Food source strain for *C. elegans*, uracil auxotrophic	15
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*q ZΔM15 Tn*10* (Tet^r)]	Stratagene
MT102	*araD139* (*ara-leu*)*7679* Δ*lac thi hsdR*	Laboratory collection
Pseudomonas putida	Bioluminescent AHL biosensor, F117 with pBBR1MCS-5	77
F117(pAS-C8)	carrying P*cepI*::*gfp*(ASV) P*lac*::*cepR*; Gm^r	
Burkholderia cenocepacia		
H111	CF isolate, Germany	78
H111-R	Km^r; *cepR*::Km mutant of H111	79
H111 *purD*	Km^r; *purD*::Km mutant of H111	This study
H111 *purF*	Km^r; *purF*::Km mutant of H111	This study
H111 *purL*	Km^r; *purL*::Km mutant of H111	This study
H111 *purA*	Km^r; *purA*::Km mutant of H111	This study
H111 *aroK*	Km^r; *aroK*::Km mutant of H111	This study
H111 *ilvC*	Km^r; *ilvC*::Km mutant of H111	This study
H111 *cysI*	Km^r; *cysI*::Km mutant of H111	This study
H111 *cysB*	Km^r; *cysB*::Km mutant of H111	This study
H111 *ahcY*	Km^r; *ahcY*::Km mutant of H111	This study
H111 *hisG*	Km^r; *hisG*::Km mutant of H111	This study
H111 *hisH*	Km^r; *hisH*::Km mutant of H111	This study
H111 *trpA*	Km^r; *trpA*::Km mutant of H111	This study
H111 *trpB*	Km^r; *trpB*::Km mutant of H111	This study
H111 *trpF*	Km^r; *trpF*::Km mutant of H111	This study
H111 *gatA*	Km^r; *gatA*::Km mutant of H111	This study
H111 *lon*	Km^r; *lon*::Km mutant of H111	This study
H111 *rsaM*	Km^r; *rsaM*::Km mutant of H111	This study
H111 *pyrD*	Km^r; *pyrD*::Km mutant of H111	This study
H111 *rsuA*	Km^r; *rsuA*::Km mutant of H111	43
G. mellonella		Fischerei Brumann, Zürich
C. elegans Bristol N2		*Caenorhabditis* Genetics Center, University of Minnesota, Minneapolis
D. melanogaster Canton-S		
Plasmids		
pRK600	Cm^r *ori*ColE1 RK2-Mob^+ RK2-Tra^+, helper plasmid in triparental conjugations	21
pSB403	Tc^r, *luxRI*′::*lux*CDABE transcriptional fusion, bioluminescent AHL sensor plasmid	80
pGEM-3Zf(+)	Ap^r, multicopy cloning vector	Promega
pUTmini-Tn*5* Km2-*luxCDABE*	Km^r, delivery vector for mini-Tn*5* Km2-*luxCDABE*	81
pCR2.1-TOPO	Ap^r, Km^r, cloning vector	Invitrogen
pRG930	16.1-kb cosmid, derived from pGV910, Sm^r/Sp^r	20
pRG930-*purD*	pRG930 containing *purD* gene, Sm^r/Sp^r	This study
pRG930-*purF*	pRG930 containing *purF* gene, Sm^r/Sp^r	This study
pRG930-*purL*	pRG930 containing *purL* gene, Sm^r/Sp^r	This study
pRG930-*pyrD*	pRG930 containing *pyrD* gene, Sm^r/Sp^r	This study
pRG930-*aroK*	pRG930 containing *aroK* gene, Sm^r/Sp^r	This study
pUC19	Cloning vector, Ap^r, *lacZ*	Invitrogen, Carlsbad, CA

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 Tn*5* 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,

TABLE 2 Primers used in this study

Primer	Sequence (5′→3′)	Description
pRG930_seq_rv	ACG TTT CAG TTT GCT CAT GG	Sequencing primer, pRG930
pRG930_seq_fw	GGT CCT GCA ACT TTA TCC G	Sequencing primer, pRG930
Kan res-v	ACC GAG GCA GTT CCA TAG G	Mini-Tn5 Km2-*luxCDABE*
Kan res-r	ATG AGC CAT ATT CAA CGG G	Mini-Tn5 Km2-*luxCDABE*
M13v	GTA AAA CGA CGG CCA G	Sequencing primer/pCR2.1TOPO
M13r	CAG GAA ACA GCT ATG AC	Sequencing primer/pCR2.1TOPO
ARB2	GGC CAC GCG TCG ACT AGT AC	Arbitrary PCR, round 2
ARB6	GGC CAC GCG TCG ACT AGT CAN NNN NNN NNN ACG CC	Arbitrary PCR, round 1
luxCext2	AGT CAT TCA ATA TTG GCA GG	Arbitrary PCR, round 1
luxCint2	GGA TTG CAC TAA ATC ATC CAC	Arbitrary PCR, round 2
Seq_O	CAC TTG TGT ATA AGA GTC AG	Sequencing primer, o end of the mini-Tn5 Km2-*luxCDABE* transposon

Southern blotting was employed. Genomic DNA was digested with SphI. 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 a 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 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, SphI 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 SphI cloning, genomic DNA was digested with SphI. 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 a *B. cenocepacia* H111 cosmid library. Chromosomal DNA was extracted from *B. cenocepacia* H111 using a standard protocol ([19]). The DNA was partially digested with EcoRI and ligated into cosmid pRG930 ([20]). Packaging and of the cosmid 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 cosmid 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 miniprep kit (Qiagen) and the cosmid ends sequenced with the primers pRG930_seq_rv and pRG930_seq_fw ([Table 2]) to determine the extent of the genomic insert.

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 multiplates (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.

***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 CO_2 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 $MgSO_4$ 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 OD_{600} of 3.0. Bacteria were collected by centrifugation at 6,500 rpm for 5 min and the supernatant filter sterilized. One hundred microliters of supernatant was mixed with 100 μl exponential-growth-phase *Pseudomonas putida* F117(pAS-C8-Gm^r). This strain is highly specific for C_8 homoserine lactone (HSL) detection. Commercial C_8 HSLs were used as a standard. The cells were incubated in FluoroNunc Polysorp microtiter plates (Nunc Roskilde) for 6 h in the dark at 30°C. Fluorescence was measured with a Lamda 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 OD_{600} of 1.0, allowed to dry, and incubated at 37°C for ~48 h.
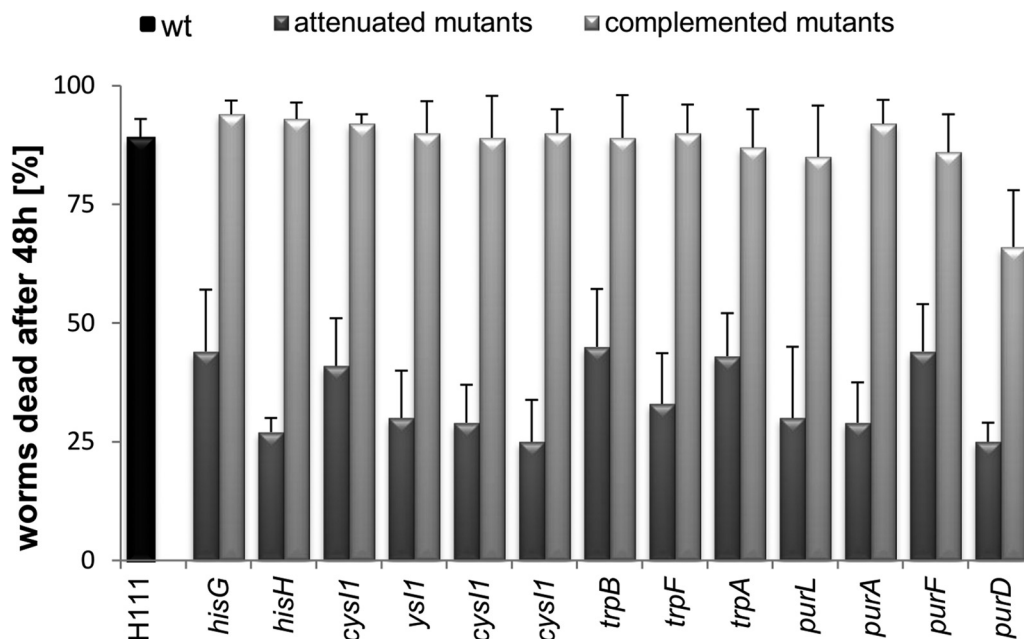
FIG 1 Virulence of wild-type *B. cenocepacia* H111 and auxotrophic mutants in the *C. elegans* model. *C. elegans* were counted 48 h after spreading the worms on medium covered with a lawn of the respective bacterial strain. Each experiment consisted of three independent replicates. Black bar represents the wild type (wt) (H111), dark gray bars represent mutants grown on NG medium, and light gray bars represent mutants grown on nutritionally supplemented NG medium. Nutritional supplementation was as follows: *hisG* and *hisH*, histidine; *cysI* and *cysB*, cysteine, *trpA*, *trpB*, and *trpF*, DL-tryptophan; *purD*, *purF*, *purL*, and *purA*, adenosine.

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 (R_fs, 0.35 and 0.37, respectively), and salicylate was visualized as a blue fluorescent band (R_f, 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 OD_{600} 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 OD_{600} 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*

Strain	Gene disrupted	H111 gene ID	Mean (SD) % dead *C. elegans* larvae after 48 h[a]	*D. melanogaster* survival time (h)	Mean (SD) % surviving *G. mellonella* after 48 h	AHL production[a]	Protease activity[a]	EPS production[a]	Siderophore production[a]	Expression of AidA[a]	Growth on ABC[a]
HIII	None (wt)		89 (4)	<72	10 (10)	+	+	+	+	+	+
A4	*purD*	358071918	24 (4)	>336	72 (18)	<	<	−	−	+	−
D9	*purF*	358073772	44 (10)	>336	29 (8)	+	<	<	+	+	+
C2-1	*purL*	358073038	30 (8)	>336	50 (20)	<	+	−	−	+	−
F8	*purA*	358073158	29 (8)	<72	0 (0)	+	+	<	<	+	−
A11	*aroK*	358076029	25 (4)	<288	93 (12)	+	+	−	+[c]	+	−
H2	*ilvC*	358071882	21 (9)	>336	27 (25)	+	+	<	−	+	−
C5	*cysI*	358073512	29 (8)	<72	NT[d]	+	+	<	+[c]	+	−
E10	*cysI*	358073512	41 (10)	<72	NT	+	+	<	+[c]	+	−
F7	*cysI*	358073512	30 (16)	<72	NT	+	+	<	+[c]	+	−
B1	*cysI*	358073512	25 (9)	<72	NT	+	+	<	+[c]	+	−
G11	*cysB*	358073513	62 (8)	<72	NT	+	+	+	+[c]	+	+
E11	*ahcY*	358071424	11 (9)	<72	NT	+	+	−	+	+	−
E8	*hisG*	358075994	44 (12)	<72	NT	+	+	<	+	+	−
G5-1	*hisH*	358075989	27 (3)	<72	NT	+	+	<	<	+	<
D1	*trpA*	358073777	43 (9)	<72	NT	+	+	−	+	−	−
G5-2	*trpB*	358073779	45 (12)	<72	NT	+	+	+	+	<	−
B8	*trpF*	358073780	33 (10)	<72	NT	<	+	−	+	+	−
C2-2	*gatA*	358076086	63 (15)	<72	NT	+	<	+	+	+	+
E5	*rsuA*	358074812	29 (9)	<72	NT	<	−	+	−	<	+
G3	*lon*	358073031	46 (10)	<72	NT	<	−	+	+	+	+
E12-2	*rsaM*	358072205	17 (8)	<72	NT	+	<	+	<	<	+
D6[b]	*pyrD*	358074754	23 (7)	>336	87 (15)	<	−	−	−	+	−
G1	*cepR*	358072206	7 (5)	<72	0 (0)	−	−	<	+	+	+

[a] +, wt level; <, less than wt level; −, absence.

[b] This strain showed a reduced growth rate in LB medium (see Fig. S1 in the supplemental material).

[c] Halo on CAS agar of normal size but pink color absent.

[d] NT, not tested.

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*, *rsuA*, 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 *rsuA* gene. An *rsuA* mutant was previously identified in a screen for biofilm-defective mutants, and this gene (previously known as *yciL*) 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$^+$ (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
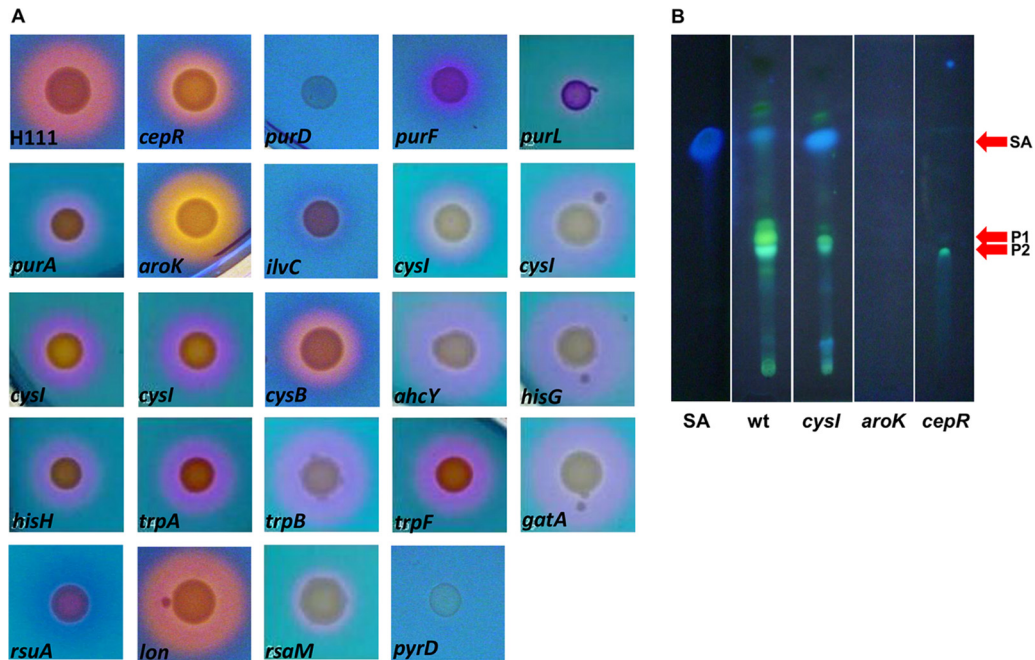
FIG 2 Siderophore production of attenuated *B. cenocepacia* H111 Tn*5* mutants. (A) Mutants were inoculated on CAS plates and incubated at 37°C for 48 h. The halo diameter corresponds to siderophore activity. A pink-tinted halo is indicative of pyochelin production. (B) Production of pyochelin as determined by thin-layer chromatography. Bands corresponding to pyochelins I and II (P1/P2) and salicylate (SA) are indicated. Extracts were as follows: SA, salicylate standard; wt, wild-type H111; *cysI*, H111 *cysI*; *aroK*, H111 *aroK*; *cepR*, H111 *cepR*.

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-
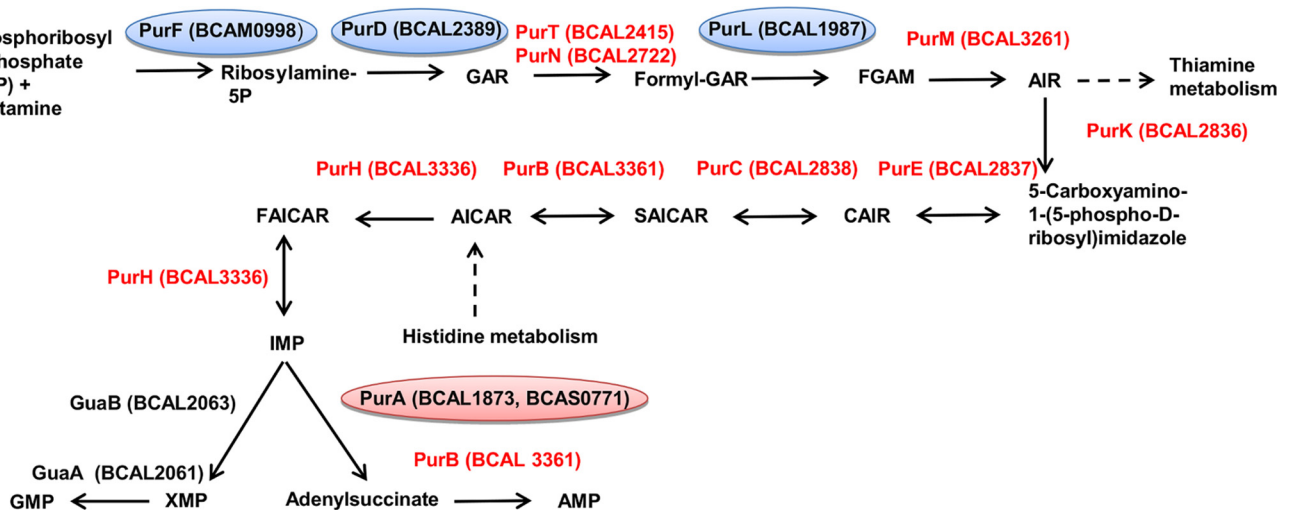

FIG 3 The *B. cenocepacia* purine biosynthetic pathway. Mutants with attenuated virulence isolated in this study are circled.
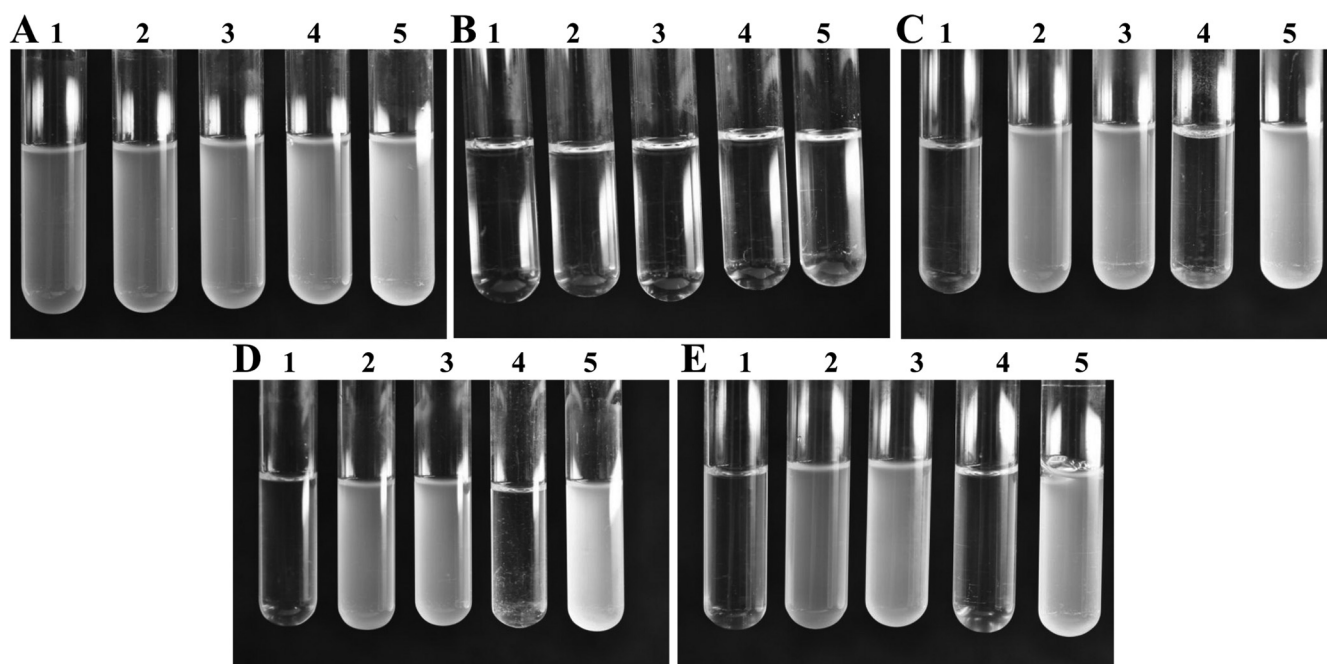
FIG 4 Growth of purine mutants in ABC medium supplemented with various metabolites. Tubes: 1, unsupplemented medium; 2, supplementation with inosine; 3, supplementation with adenine; 4, supplementation with guanine; 5, supplementation with adenine and guanine. (A) *B. cenocepacia* HIII; (B) *purA* mutant; (C) *purD* mutant; (D) *purF* mutant; (E) *purL* mutant.

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 *cysI* 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 *ahcY* (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. cenocepacia* 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
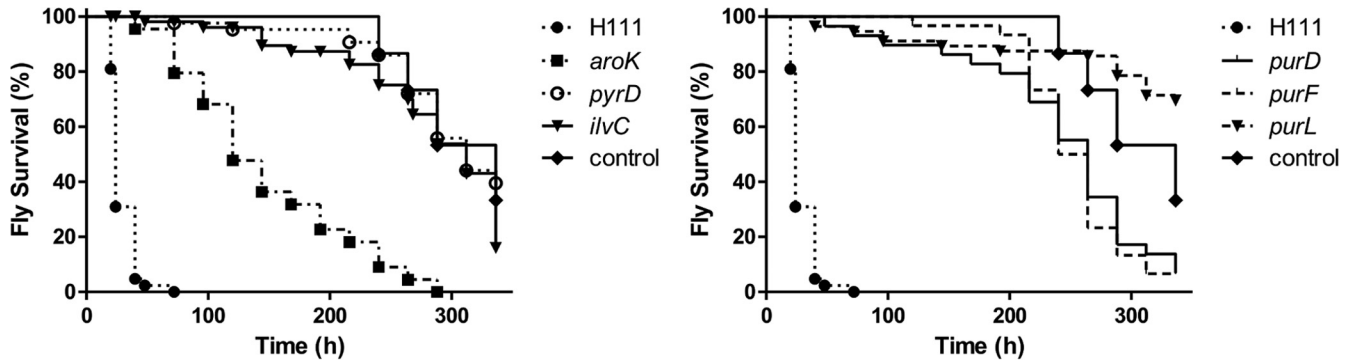
FIG 5 Virulence of wild-type *B. cenocepacia* H111 and attenuated auxotrophic Tn5 mutants in the *D. melanogaster* infection model. Fifteen flies were inoculated with bacterial culture per experiment and incubated at 26°C. Live flies were counted every 24 h postinfection. Data are based on three independent 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.

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*.
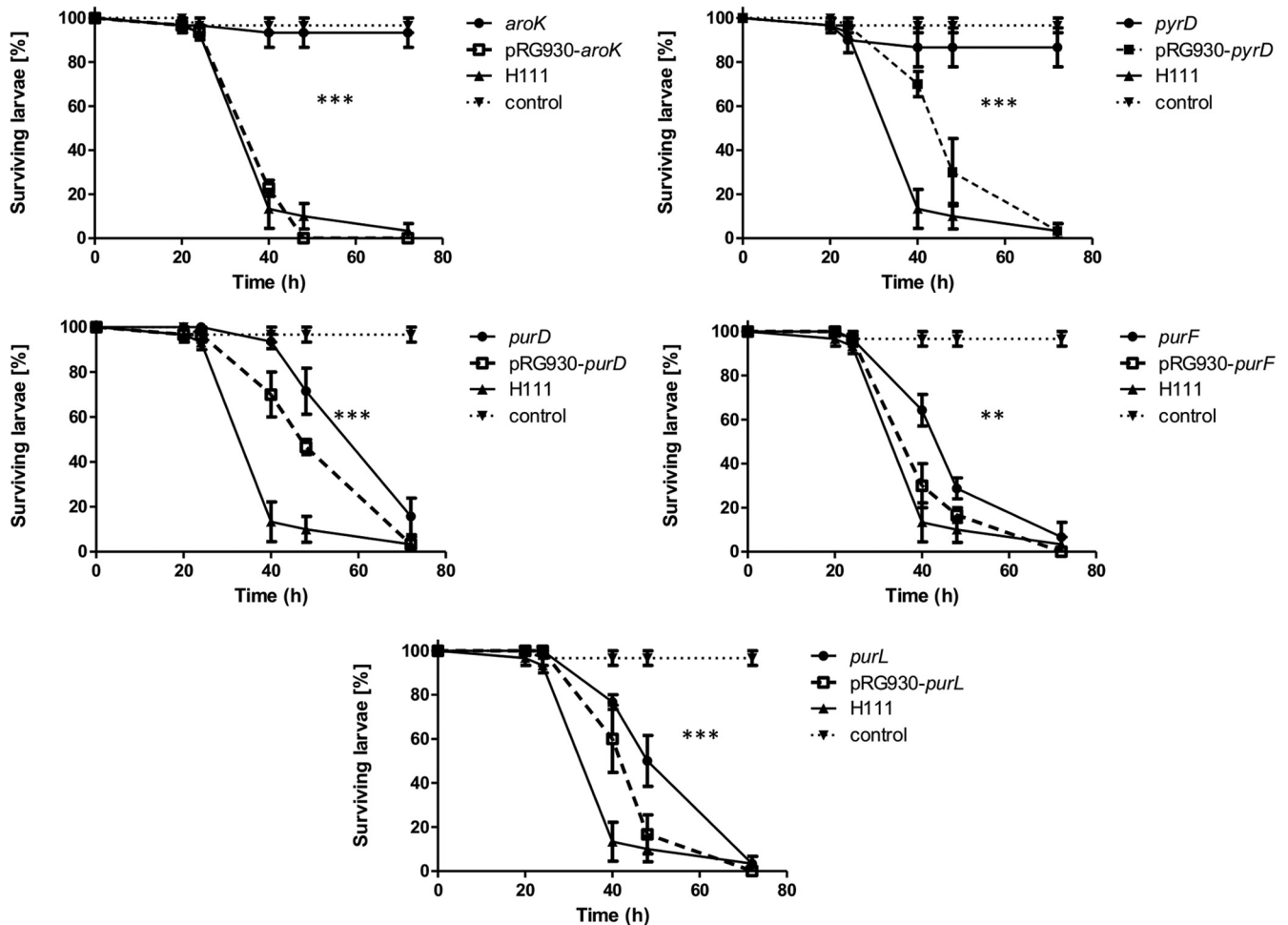

FIG 6 Virulence of wild-type *B. cenocepacia* H111type, attenuated auxotrophic Tn5 mutants, and complemented mutant strains in the *G. mellonella* infection model. *G. mellonella* larvae were infected with approximately 2×10^5 bacteria and incubated at 30°C in the dark. Live and dead larvae were counted 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: *, $0.01 \leq P \leq 0.05$; **, $0.001 \leq P \leq 0.01$; ***, $P < 0.001$.

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 550 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 the 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.

ACKNOWLEDGMENTS

REFERENCES

1. **Coenye T, Vandamme P.** 2003. Diversity and significance of *Burkholderia* species occupying diverse ecological niches. Environ. Microbiol. **5**:719–729.
2. **Eberl L, Tummler B.** 2004. *Pseudomonas aeruginosa* and *Burkholderia cepacia* in cystic fibrosis: genome evolution, interactions and adaptation. Int. J. Med. Microbiol. **294**:123–131.
3. **Mahenthiralingam E, Baldwin A, Dowson CG.** 2008. *Burkholderia cepacia* complex bacteria: opportunistic pathogens with important natural biology. J. Appl. Microbiol. **104**:1539–1551.
4. **Mahenthiralingam E, Urban TA, Goldberg JB.** 2005. The multifarious, multireplicon *Burkholderia cepacia* complex. Nat. Rev. Microbiol. **3**:144–156.
5. **Isles A, Maclusky I, Corey M, Gold R, Prober C, Fleming P, Levison H.** 1984. *Pseudomonas cepacia* infection in cystic-fibrosis—an emerging problem. J. Pediatr. **104**:206–210.
6. **Baldwin A, Sokol PA, Parkhill J, Mahenthiralingam E.** 2004. The *Burkholderia cepacia* epidemic strain marker is part of a novel genomic island encoding both virulence and metabolism-associated genes in *Burkholderia cenocepacia*. Infect. Immun. **72**:1537–1547.
7. **Loutet SA, Valvano MA.** 2010. A decade of *Burkholderia cenocepacia* virulence determinant research. Infect. Immun. **78**:4088–4100.
8. **Uehlinger S, Schwager S, Bernier SP, Riedel K, Nguyen DT, Sokol PA, Eberl L.** 2009. Identification of specific and universal virulence factors in *Burkholderia cenocepacia* strains by using multiple infection hosts. Infect. Immun. **77**:4102–4110.
9. **Castonguay-Vanier J, Vial L, Tremblay J, Deziel E.** 2010. *Drosophila melanogaster* as a model host for the *Burkholderia cepacia* complex. PLoS One **5**:e11467. doi:10.1371/journal.pone.0011467.
10. **Apidianakis Y, Rahme LG.** 2009. Drosophila melanogaster as a model host for studying *Pseudomonas aeruginosa* infection. Nat. Protoc. **4**:1285–1294.
11. **Seed KD, Dennis JJ.** 2008. Development of *Galleria mellonella* as an alternative infection model for the *Burkholderia cepacia* complex. Infect. Immun. **76**:1267–1275.
12. **Huber B, Feldmann F, Kothe M, Vandamme P, Wopperer J, Riedel K, Eberl L.** 2004. Identification of a novel virulence factor in *Burkholderia cenocepacia* H111 required for efficient slow killing of *Caenorhabditis elegans*. Infect. Immun. **72**:7220–7230.
13. **Bertani G.** 1951. Studies on lysogenesis. 1. The mode of phage liberation by lysogenic *Escherichia coli*. J. Bacteriol. **62**:293–300.
14. **Tan MW, Rahme LG, Sternberg JA, Tompkins RG, Ausubel FM.** 1999. *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. Proc. Natl. Acad. Sci. U. S. A. **96**:2408–2413.
15. **Brenner S.** 1974. Genetics of *Caenorhabditis elegans*. Genetics **77**:71–94.
16. **Sambrook J, Fritsch EF, Maniatis T.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
17. **Southern EM.** 1975. Detection of specific sequences among DNA fragments separated by gel-electrophoresis. J. Mol. Biol. **98**:503–517.
18. **O'Toole GA, Kolter R.** 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. **28**:449–461.
19. **Better M, Lewis B, Corbin D, Ditta G, Helinski DR.** 1983. Structural relationships among *Rhizobium meliloti* symbiotic promoters. Cell **35**:479–485.
20. **Vandeneede G, Deblaere R, Goethals K, Vanmontagu M, Holsters M.** 1992. Broad host range and promoter selection vectors for bacteria that interact with plants. Mol. Plant Microbe Interact. **5**:228–234.
21. **de Lorenzo V, Timmis KN.** 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn*5*-derived and Tn*10*-derived minitransposons. Methods Enzymol. **235**:386–405.
22. **Köthe M, Antl M, Huber B, Stoecker K, Ebrecht D, Steinmetz I, Eberl L.** 2003. Killing of *Caenorhabditis elegans* by *Burkholderia cepacia* is controlled by the *cep* quorum-sensing system. Cell. Microbiol. **5**:343–351.
23. **Cardona ST, Wopperer J, Eberl L, Valvano MA.** 2005. Diverse pathogenicity of *Burkholderia cepacia* complex strains in the *Caenorhabditis elegans* host model. FEMS Microbiol. Lett. **250**:97–104.
24. **Stiernagle T, Hope IA (ed).** 1999. *C. elegans*: a practical approach. Oxford University Press, New York, NY.
25. **Schwyn B, Neilands JB.** 1987. Universal chemical-assay for the detection and determination of siderophores. Anal. Biochem. **160**:47–56.
26. **Lewenza S, Conway B, Greenberg EP, Sokol PA.** 1999. Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI. J. Bacteriol. **181**:748–756.
27. **Visca P, Ciervo A, Sanfilippo V, Orsi N.** 1993. Iron-regulated salicylate synthesis by *Pseudomonas* spp. J. Gen. Microbiol. **139**:1995–2001.
28. **Rinehart KL, Staley AL, Wilson SR, Ankenbauer RG, Cox CD.** 1995. Stereochemical assignment of the pyochelins. J. Org. Chem. **60**:2786–2791.
29. **Ankenbauer RG, Toyokuni T, Staley A, Rinehart KL, Jr, Cox CD.** 1988. Synthesis and biological activity of pyochelin, a siderophore of *Pseudomonas aeruginosa*. J. Bacteriol. **170**:5344–5351.
30. **Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Phillips GB (ed).** 1981. Manual of methods for general bacteriology. American Society for Microbiology, Washington, DC.
31. **Sage A, Linker A, Evans LR, Lessie TG.** 1990. Hexose phosphate-metabolism and exopolysaccharide formation in *Pseudomonas cepacia*. Curr. Microbiol. **20**:191–198.
32. **Clark DJ, Maaloe O.** 1967. DNA replication and division cycle in *Escherichia coli*. J. Mol. Biol. **23**:99–112.
33. **Sokol PA, Darling P, Woods DE, Mahenthiralingam E, Kooi C.** 1999. Role of ornibactin biosynthesis in the virulence of *Burkholderia cepacia*: characterization of *pvdA*, the gene encoding L-ornithine N-5-oxygenase. Infect. Immun. **67**:4443–4455.
34. **Visser MB, Majumdar S, Hani E, Sokol PA.** 2004. Importance of the ornibactin and pyochelin siderophore transport systems in *Burkholderia cenocepacia* lung infections. Infect. Immun. **72**:2850–2857.
35. **Malott RJ, O'Grady EP, Toller J, Inhuelsen S, Eberl L, Sokol PA.** 2009. A *Burkholderia cenocepacia* orphan LuxR homolog is involved in quorum-sensing regulation. J. Bacteriol. **191**:2447–2460.
36. **Chung JW, Altman E, Beveridge TJ, Speert DP.** 2003. Colonial morphology of *Burkholderia cepacia* complex genomovar III: implications in exopolysaccharide production, pilus expression, and persistence in the mouse. Infect. Immun. **71**:904–909.
37. **Cunha MV, Sousa SA, Leitao JH, Moreira LM, Videira PA, SA-Correia I.** 2004. Studies on the involvement of the exopolysaccharide produced by cystic fibrosis-associated isolates of the *Burkholderia cepacia* complex in biofilm formation and in persistence of respiratory infections. J. Clin. Microbiol. **42**:3052–3058.
38. **Bartholdson SJ, Brown AR, Mewburn BR, Clarke DJ, Fry SC, Campopiano DJ, Govan JR.** 2008. Plant host and sugar alcohol induced exopolysaccharide biosynthesis in the *Burkholderia cepacia* complex. Microbiology **154**:2513–2521.
39. **Ferreira AS, Leitao JH, Silva IN, Pinheiro PF, Sousa SA, Ramos CG, Moreira LM.** 2010. Distribution of cepacian biosynthesis genes among environmental and clinical *Burkholderia* strains and role of cepacian exopolysaccharide in resistance to stress conditions. Appl. Environ. Microbiol. **76**:441–450.
40. **Conway BA, Chu KK, Bylund J, Altman E, Speert DP.** 2004. Production of exopolysaccharide by *Burkholderia cenocepacia* results in altered cell-

surface interactions and altered bacterial clearance in mice. J. Infect. Dis. **190:**957–966.

41. **Sousa SA, Ulrich M, Bragonzi A, Burke M, Worlitzsch D, Leitao JH, Meisner C, Eberl L, SA-Correia I, Doring G.** 2007. Virulence of *Burkholderia cepacia* complex strains in gp91phox−/− mice. Cell. Microbiol. **9:**2817–2825.

42. **Agnoli K, Schwager S, Uehlinger S, Vergunst A, Viteri DF, Nguyen DT, Sokol PA, Carlier A, Eberl L.** 2012. Exposing the third chromosome of *Burkholderia cepacia* complex strains as a virulence plasmid. Mol. Microbiol. **83:**362–378.

43. **Huber B, Riedel K, Kothe M, Givskov M, Molin S, Eberl L.** 2002. Genetic analysis of functions involved in the late stages of biofilm development in *Burkholderia cepacia* H111. Mol. Microbiol. **46:**411–426.

44. **Van Melderen L, Aertsen A.** 2009. Regulation and quality control by Lon-dependent proteolysis. Res. Microbiol. **160:**645–651.

45. **Snider J, Thibault G, Houry WA.** 2008. The AAA plus superfamily of functionally diverse proteins. Genome Biology **9:**216.

46. **Takaya A, Suzuki M, Matsui H, Tomoyasu T, Sashinami H, Nakane A, Yamamoto T.** 2003. Lon, a stress-induced ATP-dependent protease, is critically important for systemic *Salmonella enterica* serovar Typhimurium infection of mice. Infect. Immun. **71:**690–696.

47. **Lan L, Deng X, Xiao Y, Zhou J-M, Tang X.** 2007. Mutation of Lon protease differentially affects the expression of *Pseudomonas syringae* type III secretion system genes in rich and minimal media and reduces pathogenicity. Mol. Plant Microbe Interact. **20:**682–696.

48. **Cohn MT, Ingmer H, Mulholland F, Jorgensen K, Wells JM, Brondsted L.** 2007. Contribution of conserved ATP-dependent proteases of *Campylobacter jejuni* to stress tolerance and virulence. Appl. Environ. Microbiol. **73:**7803–7813.

49. **Su SC, Stephens BB, Alexandre G, Farrand SK.** 2006. Lon protease of the alpha-proteobacterium *Agrobacterium tumefaciens* is required for normal growth, cellular morphology and full virulence. Microbiology **152:**1197–1207.

50. **Robertson GT, Kovach ME, Allen CA, Ficht TA, Roop RM.** 2000. The *Brucella abortus* Lon functions as a generalized stress response protease and is required for wild-type virulence in BALB/c mice. Mol. Microbiol. **35:**577–588.

51. **Butler SM, Festa RA, Pearce MJ, Darwin KH.** 2006. Self-compartmentalized bacterial proteases and pathogenesis. Mol. Microbiol. **60:**553–562.

52. **Tsilibaris V, Maenhaut-Michel G, Van Melderen L.** 2006. Biological roles of the Lon ATP-dependent protease. Res. Microbiol. **157:**701–713.

53. **Zhang Y, Morar M, Ealick SE.** 2008. Structural biology of the purine biosynthetic pathway. Cell. Mol. Life Sci. **65:**3699–3724.

54. **Jenkins A, Cote C, Twenhafel N, Merkel T, Bozue J, Welkos S.** 2011. Role of purine biosynthesis in *Bacillus anthracis* pathogenesis and virulence. Infect. Immun. **79:**153–166.

55. **Corbett CR, Burtnick MN, Kooi C, Woods DE, Sokol PA.** 2003. An extracellular zinc metalloprotease gene of *Burkholderia cepacia*. Microbiology **149:**2263–2271.

56. **Kooi C, Subsin B, Chen R, Pohorelic B, Sokol PA.** 2006. *Burkholderia cenocepacia* ZmpB is a broad-specificity zinc metalloprotease involved in virulence. Infect. Immun. **74:**4083–4093.

57. **Kooi C, Corbett CR, Sokol PA.** 2005. Functional analysis of the *Burkholderia cenocepacia* ZmpA metalloprotease. J. Bacteriol. **187:**4421–4429.

58. **Farmer KL, Thomas MS.** 2004. Isolation and characterization of *Burkholderia cenocepacia* mutants deficient in pyochelin production: pyochelin biosynthesis is sensitive to sulfur availability. J. Bacteriol. **186:**270–277.

59. **Meibom KL, Charbit A.** 2010. *Francisella tularensis* metabolism and its relation to virulence. Front. Microbiol. **1:**140.

60. **Hoiseth SK, Stocker BAD.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature **291:**238–239.

61. **Mei JM, Nourbakhsh F, Ford CW, Holden DW.** 1997. Identification of *Staphylococcus aureus* virulence genes in a murine model of bacteraemia using signature-tagged mutagenesis. Mol. Microbiol. **26:**399–407.

62. **Polissi A, Pontiggia A, Feger G, Altieri M, Mottl H, Ferrari L, Simon D.** 1998. Large-scale identification of virulence genes from *Streptococcus pneumoniae*. Infect. Immun. **66:**5620–5629.

63. **Brubaker RR.** 1970. Interconversion of purine mononucleotides in *Pasteurella pestis*. Infect. Immun. **1:**446–454.

64. **Kim YR, Lee SE, Kim CM, Kim SY, Shin EK, Shin DH, Chung SS, Choy HE, Progulske-Fox A, Hillman JD, Handfield M, Rhee JH.** 2003. Characterization and pathogenic significance of *Vibrio vulnificus* antigens preferentially expressed in septicemic patients. Infect. Immun. **71:**5461–5471.

65. **Ivanovics G, Marjai E, Dobozy A.** 1968. The growth of purine mutants of *Bacillus anthracis* in the body of the mouse. J. Gen. Microbiol. **53:**147–162.

66. **Crawford RM, Van De Verg L, Yuan L, Hadfield TL, Warren RL, Drazek ES, Houng HH, Hammack C, Sasala K, Polsinelli T, Thompson J, Hoover DL.** 1996. Deletion of *purE* attenuates *Brucella melitensis* infection in mice. Infect. Immun. **64:**2188–2192.

67. **Alcantara RB, Read RDA, Valderas MW, Brown TD, Roop RM.** 2004. Intact purine biosynthesis pathways are required for wild-type virulence of *Brucella abortus* 2308 in the BALB/c mouse model. Infect. Immun. **72:**4911–4917.

68. **Lee SE, Kim SY, Kim CM, Kim MK, Kim YR, Jeong K, Ryu HJ, Lee YS, Chung SS, Choy HE, Rhee JH.** 2007. The *pyrH* gene of *Vibrio vulnificus* is an essential *in vivo* survival factor. Infect. Immun. **75:**2795–2801.

69. **Klarsfeld AD, Goossens PL, Cossart P.** 1994. Five *Listeria monocytogenes* genes preferentially expressed in infected mammalian cells: *plcA*, *purH*, *purD*, *pyrE* and an arginine ABC transporter gene, *arpJ*. Mol. Microbiol. **13:**585–597.

70. **Samant S, Lee H, Ghassemi M, Chen J, Cook JL, Mankin AS, Neyfakh AA.** 2008. Nucleotide biosynthesis is critical for growth of bacteria in human blood. PLoS Pathog. **4:**e37. doi:10.1371/journal.ppat.0040037.

71. **Gunel-Ozcan A, Brown KA, Allen AG, Maskell DJ.** 1997. *Salmonella typhimurium aroB* mutants are attenuated in BALB/c mice. Microb. Pathog. **23:**311–316.

72. **Priebe GP, Meluleni GJ, Coleman FT, Goldberg JB, Pier GB.** 2003. Protection against fatal *Pseudomonas aeruginosa* pneumonia in mice after nasal immunization with a live, attenuated *aroA* deletion mutant. Infect. Immun. **71:**1453–1461.

73. **Stritzker J, Janda J, Schoen C, Taupp M, Pilgrim S, Gentschev I, Schreier P, Geginat G, Goebel W.** 2004. Growth, virulence, and immunogenicity of *Listeria monocytogenes aro* mutants. Infect. Immun. **72:**5622–5629.

74. **Cuccui J, Easton A, Chu KK, Bancroft GJ, Oyston PCF, Titball RW, Wren BW.** 2007. Development of signature-tagged mutagenesis in *Burkholderia pseudomallei* to identify genes important in survival and pathogenesis. Infect. Immun. **75:**1186–1195.

75. **Roberts F, Roberts CW, Johnson JJ, Kyle DE, Krell T, Coggins JR, Coombs GH, Milhous WK, Tzipori S, Ferguson DJP, Chakrabarti D, McLeod R.** 1998. Evidence for the shikimate pathway in apicomplexan parasites. Nature **393:**801–805.

76. **Coggins JR, Abell C, Evan LB, Frederickson M, Robinson DA, Roszak AW, Lapthorn AP.** 2003. Experiences with the shikimate-pathway enzymes as targets for rational drug design. Biochem. Soc. Trans. **31:**548–552.

77. **Steidle A, Sigl K, Schuhegger R, Ihring A, Schmid M, Gantner S, Stoffels M, Riedel K, Givskov M, Hartmann A, Langebartels C, Eberl L.** 2001. Visualization of N-acylhomoserine lactone-mediated cell-cell communication between bacteria colonizing the tomato rhizosphere. Appl. Environ. Microbiol. **67:**5761–5770.

78. **Romling U, Fiedler B, Bosshammer J, Grothues D, Greipel J, von der Hardt H, Tummler B.** 1994. Epidemiology of chronic *Pseudomonas aeruginosa* infections in cystic fibrosis. J. Infect. Dis. **170:**1616–1621.

79. **Huber B, Riedel K, Hentzer M, Heydorn A, Gotschlich A, Givskov M, Molin S, Eberl L.** 2001. The *cep* quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. Microbiology **147:**2517–2528.

80. **Winson MK, Swift S, Fish L, Throup JP, Jorgensen F, Chhabra SR, Bycroft BW, Williams P, Stewart G.** 1998. Construction and analysis of *luxCDABE*-based plasmid sensors for investigating N-acyl homoserine lactone-mediated quorum sensing. FEMS Microbiol. Lett. **163:**185–192.

81. **Winson MK, Swift S, Hill PJ, Sims CM, Griesmayr G, Bycroft BW, Williams P, Stewart G.** 1998. Engineering the *luxCDABE* genes from *Photorhabdus luminescens* to provide a bioluminescent reporter for constitutive and promoter probe plasmids and mini-Tn*5* constructs. FEMS Microbiol. Lett. **163:**193–202.