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σ^{54}-Dependent Response to Nitrogen Limitation and Virulence in *Burkholderia cenocepacia* Strain H111

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Members of the genus *Burkholderia* are versatile bacteria capable of colonizing highly diverse environmental niches. In this study, we investigated the global response of the opportunistic pathogen *Burkholderia cenocepacia* H111 to nitrogen limitation at the transcript and protein expression levels. In addition to a classical response to nitrogen starvation, including the activation of glutamine synthetase, PII proteins, and the two-component regulatory system NtrBC, *B. cenocepacia* H111 also upregulated polyhydroxybutyrate (PHB) accumulation and exopolysaccharide (EPS) production in response to nitrogen shortage. A search for consensus sequences in promoter regions of nitrogen-responsive genes identified a σ^{54} consensus sequence. The mapping of the σ^{54} regulon as well as the characterization of a σ^{54} mutant suggests an important role of σ^{54} not only in control of nitrogen metabolism but also in the virulence of this organism.

Members of the genus *Burkholderia* are versatile bacteria with diverse metabolic capacities which allow them to successfully compete in diverse environments (1). *Burkholderia cenocepacia* belongs to the *Burkholderia cepacia* complex (*Bcc*), which is a group of species that comprises opportunistic pathogens that can cause severe infections in cystic fibrosis (CF) and immunocompromised patients (2). During CF lung infection, bacteria face an environment with a heterogeneous distribution of oxygen and nutrients, as well as high concentrations of antimicrobials. Several studies have shown that bacteria experience nutrient limitation during chronic lung infection (3–6). Nitrogen is a major nutrient for cells, and nitrogen metabolism and its regulation have been studied in several bacterial species (7, 8). Ammonium is the preferred nitrogen source for most bacteria; nitrogen assimilation involves the enzymes glutamate dehydrogenase (GDH; encoded by *gdhA*), glutamine synthetase (GS; encoded by *glnA*), and glutamate synthase (GOGAT; encoded by *gltAB*). In *Escherichia coli*, as well as the majority of bacteria studied so far, glutamine is the main intracellular signal for nitrogen availability (9). The global nitrogen regulatory system (Ntr) has been shown to coordinate the expression of key enzymes in nitrogen metabolism in several bacteria (10). In enteric bacteria, the Ntr system consists of four enzymes: a uridyltransferase/uridylyl-removing enzyme (UTase/UR; encoded by *glnD*), the small trimeric signaling protein PII (encoded by *glnB*), which is modified by the bifunctional UTase/UR, and a two-component regulatory system composed of the histidine protein kinase NtrB and the transcriptional regulator NtrC. NtrC is an enhancer-binding protein (EBP) that activates transcription of σ^{54}-containing RNA polymerase complexes. Under nitrogen-limited conditions, the intracellular level of glutamine decreases, thereby activating the kinase activity of NtrB, which in turn leads to phosphorylation of NtrC. Phosphorylated NtrC activates transcription at promoters dependent on the alternative sigma factor σ^{54} (also referred to as RpoN), which binds to conserved short sequences that are located at positions −24 and −12 of the transcription initiation site (GG and GC, respectively) (11, 12). While σ^{54} was originally identified as a sigma factor required to transcribe genes involved in nitrogen metabolism (11, 13), it was later shown to also control a wide range of biological functions, including motility, biofilm formation (3, 14), the type III secretion system, and alginate synthesis (15–18), by utilizing specific EBPs.

However, it is possible that different ecological habitats of bacteria determine differences between the Ntr systems. In this study, we investigated the global response of *B. cenocepacia* H111 to nitrogen limitation, a relevant condition in different environments, at both the transcript and protein levels. Among the genes upregulated by nitrogen limitation, we found several genes important for nitrogen metabolism as well as genes involved in other cellular functions, such as polyhydroxybutyrate (PHB) accumulation and exopolysaccharide (EPS) production. A search for consensus sequences in the promoter regions of regulated genes showed an enrichment of σ^{54} boxes, underlining an important role of this alternative sigma factor in the response of *B. cenocepacia* to nitrogen limitation. Mutation of σ^{54} in *B. cenocepacia* H111 and mapping of the σ^{54}-dependent regulon revealed an important role for this sigma factor not only in the control of nitrogen assimilation

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genes but also in the control of genes involved in EPS production, biofilm formation, motility, and virulence.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains and plasmids used in this work are listed in Table S1 in the supplemental material. *E. coli* and *B. cenocepacia* H111 cells were routinely cultivated in Luria-Bertani medium (19) at 37°C, using the following antibiotics at the indicated concentrations: ampicillin (100 μg/ml for *E. coli*), kanamycin (30 μg/ml for *E. coli* and 50 μg/ml for H111), chloramphenicol (20 μg/ml for *E. coli* and 80 μg/ml for H111), and gentamicin (10 μg/ml). Cultures for transcriptome analysis were cultivated in defined buffered AB minimal medium (20) with 10 mM sodium citrate as the carbon source. To create nitrogen-replete conditions, 15 mM ammonium chloride (NH_4Cl) was used, while 0.3 mM NH_4Cl was used to initiate nitrogen-limited conditions. Cultures were grown at 37°C with agitation (220 rpm), using 500-ml Erlenmeyer flasks containing 100 ml of medium. For each strain or condition, the growth of three independent cultures was analyzed. To assess growth using different nitrogen sources, 7.5 mM urea, 15 mM nitrate, and the amino acids serine (15 mM), agmatine (3.75 mM), and ornithine (7.5 mM) were tested.

RNA-Seq and data analysis. Total RNA from H111 grown with 15 mM NH_4Cl (replete conditions) or 0.3 mM NH_4Cl (starvation conditions) in ABC minimal medium to the end of the exponential growth phase (optical density at 600 nm [OD_{600}] of 0.8 or 0.4, respectively) was extracted using a modified hot acid phenol protocol (21). For analysis of σ^{54} mutant and complemented strains, cells were grown with 15 mM NH_4Cl to exponential phase and, after washing, were further incubated for 1 h under starving conditions. The complete removal of genomic DNA by use of DNase I (Promega) was verified by a PCR with 40 cycles. Samples were further purified using an RNeasy kit (Qiagen), RNA quality was checked using RNA nanochips (RNA integrity number [RIN] of >8; Agilent 2100 bioanalyzer), and rRNA was depleted using two rounds of MICROBExpress treatment, following the instructions provided by the manufacturer (Ambion, Life Technologies, Foster City, CA). The remaining mRNA was quantified, and 500 ng was used for first- and second-strand cDNA synthesis and library preparation, using an Ovation Complete prokaryotic transcriptome sequencing (RNA-Seq) library system (Nugen). The cDNA libraries were purified using an Agencourt AMPure XP kit (Beckman Coulter Genomics) and were analyzed by capillary electrophoresis using a high-fidelity DNA chip from Agilent (size range, 100 to 800 bp). Illumina single-end sequencing was performed on a HiSeq2000 instrument. The sequence reads were processed and then mapped to the recently finished H111 genome sequence (accession no. HG938370, HG938371, and HG938372) (22), using CLC Genomics Workbench v7.0 (CLC Bio) and allowing up to 2 mismatches per read. The mapped reads (or spectral counts in the case of protein expression data [see below]) were analyzed for differential expression by using DESeq software (23).

Proteomic experiments and data analysis. Sample preparation and mass spectrometry (MS) analysis were performed as previously described (24). In brief, proteins extracted from cytoplasmic or total membrane fractions of *B. cenocepacia* grown in ABC medium with either replete (15 mM) or limited (0.3 mM) nitrogen levels were first separated in a Tris-HCl-polyacrylamide gel. After reduction and carbamidomethylation, the proteins were digested with trypsin (Promega, Madison, WI), and the resulting peptides were separated by reverse-phase high-pressure liquid chromatography (RP-HPLC) and analyzed by use of an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA) interfaced with a nanoelectrospray source (24). Mass spectra were further processed with an in-house processing pipeline (25) that extracts fragment ion mass spectra from Thermo raw files by using msconvert (ProteoWizard, version 3.0.3831) and searches for matching peptides in a *B. cenocepacia* H111 protein database also containing 256 common contaminants (e.g., human keratin and trypsin), using the powerful search engine MS-GF+ (v9979) (25). The same parameters as those described previously were used (26). Using the decoy option of MS-GF+, the list of peptide spectrum matches (PSMs) was filtered to an estimated overall false discovery rate (FDR) of 0.5%. The FDR at the protein level amounted to about 2.8% when requiring two unambiguous peptides (class 1a or 3a, based on a PeptideClassifier analysis [27]) or three spectra for protein identification under either condition. A total of 2,854 or 2,792 proteins (3,215 overall) was identified under nitrogen-replete or -starved conditions, respectively.

Functional analysis. To be able to rely on a rich source of functional annotations, we carried out a reciprocal best-BLAST-hit analysis of the *B. cenocepacia* H111 proteins against all *B. cenocepacia* strain J2315 proteins by using Proteinortho (v5.11) (28). For hits with E values of $<10^{10}$, we carried over the EggNOG annotation that is available for *B. cenocepacia* J2315 (29) to the H111 orthologs. For operon prediction, we transferred the prediction that was made using the Prokaryotic Operon DataBase (ProOpDB) (30) for J2315 to the respective orthologs in H111.

Genome-wide sequence motif prediction. The publicly available software RSAT (http://rsat.ulb.ac.be/) was used to search for consensus sequences of σ^{54} binding sites, taking the 200 nucleotides (nt) upstream of the start codons of regulated genes and the position-specific frequency matrix (PSFM) published by Dombrech et al. (31) as inputs. Only consensus sequences below a stringent threshold ($P \leq 0.00005$) were considered, resulting in 107 genes/operons with potential σ^{54}-dependent promoters in the *B. cenocepacia* H111 genome (see Table S2 in the supplemental material). The list of 107 genes downstream of the predicted promoters scoring over the selected threshold includes several of the paradigms for σ^{54}-dependent transcription, such as *glnA*, *ntrB*, *ureA*, the urea transporter gene I35_0775, and the assimilatory nitrate reductase gene cluster. The position-specific scoring matrix (PSSM) for *B. cenocepacia* H111 (see Table S5) was calculated using RSAT (http://rsat.ulb.ac.be/).

Construction of *B. cenocepacia* H111 mutant strains. Chromosomal DNAs of *B. cenocepacia* strains were isolated by the Sarkosyl-pronase method (32). Plasmid DNAs from *E. coli* strains were obtained by using a NucleoSpin plasmid kit (Macherey-Nagel, Düren, Germany). To generate *B. cenocepacia* H111-σ^{54} (an insertional mutant in I35_2858, the ortholog of *B. cenocepacia* J2315 BCAL0813), a 507-bp internal fragment of I35_2858 was amplified with GoTaq polymerase (Promega), using primers CA90 and CA91 (see Table S1 in the supplemental material). The PCR product was cloned into pGEM-T Easy (Promega) and then subcloned into pSHAFT2 as a NotI fragment, generating pSHAFT2-σ^{54}. The resulting plasmid was mobilized into the *B. cenocepacia* H111 wild type by triparental mating. The correct genomic integration was verified by PCR using oligonucleotides CA94_check and pSHAFT_check. To complement H111-σ^{54}, the complete I35_2858 open reading frame (ORF) was amplified with *Pfu* polymerase (Promega) by using oligonucleotides CA235_rpoN-F_HindIII and CA237_rpoN-R_BamHI (see Table S1). The PCR product was purified by use of a QIAquick gel extraction kit (Qiagen) and then digested with HindIII and BamHI. The digested PCR product was purified by use of a QIAquick PCR purification kit (Qiagen) and then cloned into the corresponding sites of pBBR1MSC-5, thus creating pBBR1-σ^{54}. The integrity of the I35_2858 ORF in the complementation vector was verified by DNA sequencing. The complementing vector was mobilized into *B. cenocepacia* H111-σ^{54} by triparental mating.

Phenotypic analysis. To induce PHB production, bacteria were grown on plates containing ABC medium containing either 15 mM or 0.3 mM NH_4Cl for 2 days at 37°C. Cells were suspended in 0.9% NaCl and subsequently stained with Nile blue, as described previously (33). The bacterial cells were subsequently stained with 334 μM Syto 9 (Life Technologies) for 10 min at room temperature. The PHB-Nile blue complexes were visualized at 550/580 nm (absorption/fluorescence emission wavelengths), and bacterial cell-Syto 9 complexes were visualized at 485/496 nm (absorption/fluorescence emission wavelengths). Microscopic inspections and image acquisition were performed on a confocal laser scanning microscope (CLSM) (DM5500Q; Leica) equipped with a ×63/1.4 or ×100/1.44 oil objective. Captured images

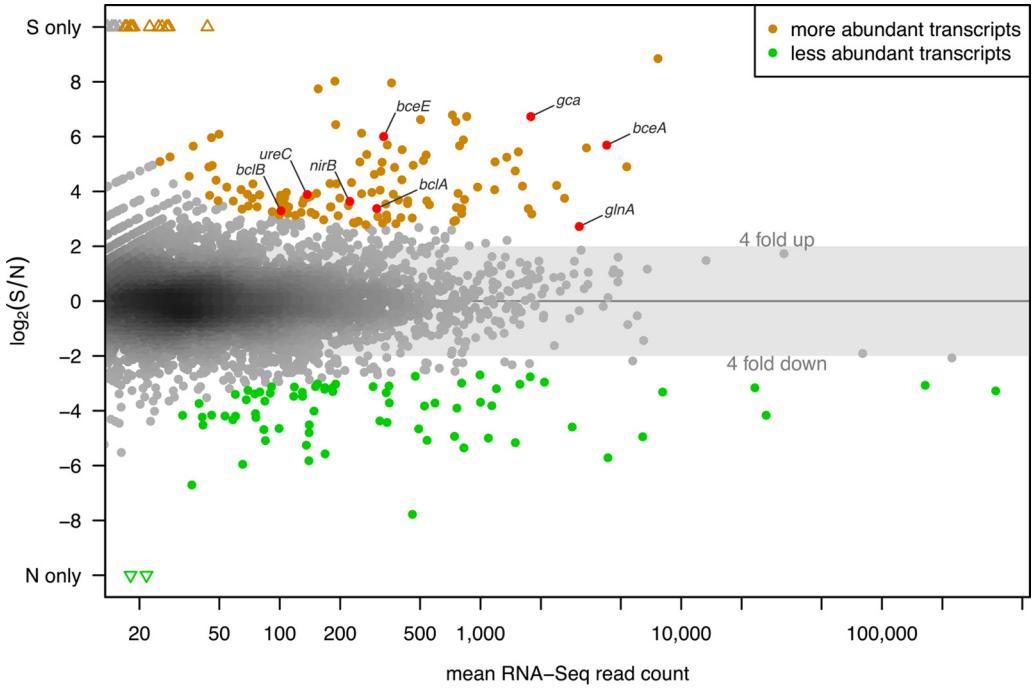

FIG 1 Differential transcript expression under nitrogen-limited and -replete conditions. The MA plot (M = log ratios; A = averages) shows the $\log_2$ fold changes in transcript expression in *B. cenocepacia* H111 grown under nitrogen-limited (S) versus nitrogen-replete (N) conditions. The 201 regulated genes ($P < 0.12$) are shown in color: genes with increased expression under nitrogen-limited conditions are indicated in orange, and downregulated genes are shown in green. Genes of particular interest are labeled.

were further analyzed with the Leica Application Suite (Mannheim, Germany) and Imaris 8.0 software (Bitplane). Biofilm formation was quantified in a microtiter dish assay as described by Huber et al. (34). EPS production was assessed on modified YEM medium plates (0.4% mannitol, 0.05% yeast extract) (35). Swarming and swimming activities were tested by inoculating cells onto plates containing ABC medium supplemented with 0.1% Casamino Acids that were solidified with 0.4% and 0.2% agar, respectively (36). Plates were incubated for 2 days at 37°C.

qPCR analysis. The expression of *B. cenocepacia* H111 genes I35_4767 (*bceA*) and I35_4923, both of which are involved in cepacian synthesis, I35_6025 (*bapA*), I35_4183 (*bclC*), I35_2151 (*glnA*), and I35_0767 (*ureC*) was analyzed by quantitative reverse transcription-PCR (qRT-PCR) using Brilliant III Ultra-Fast SYBR green QPCR master mix (Agilent, Switzerland) and an Mx3000P instrument (Agilent, Switzerland). cDNAs were prepared from biological replicates as previously described (37). Each PCR was run in triplicate with 3 dilutions of cDNA (15, 7.5, and 3.75 ng), using 15 μl 2× Brilliant III Ultra-Fast SYBR green QPCR master mix and 5 μM (each) individual primers in a total volume of 24 μl. Fold changes in expression were calculated using the $\Delta\Delta C_T$ method (38). The primary σ factor gene *rpoD* (I35_4837) was used as a reference for normalization. The primers used are listed in Table S1 in the supplemental material.

Pathogenicity assay using *Caenorhabditis elegans*. Analysis of toxicity from bacterial strains toward *C. elegans* was performed using a modified protocol described by Künzler et al. (39). The *C. elegans pmk-1*(*km25*) strain (kindly provided by M. Künzler) was maintained on nematode growth medium (NGM) and fed with *E. coli* OP50 as described previously (40). In order to yield a synchronous population of L1 larvae, an egg preparation of gravid worms was obtained as described previously (41). Eggs were incubated on unseeded NGM plates overnight at 20°C and grown to larval stage L1. The L1 larvae were washed from the plate by use of phosphate-buffered saline (PBS) and collected in an Eppendorf tube. The same day, an overnight culture of a bacterial strain was centrifuged for 2.5 min at 5,000 rpm, washed with PBS, and adjusted in PBS to an OD_{600} of 2.0. Eighty microliters of each bacterial culture was mixed with 20 μl of sample containing 15 to 30 nematodes in a 96-well plate. The plate was incubated at 20°C in the dark. After 48 h, the developmental stages of the worms were evaluated. Data shown are means for four biological replicates.

Accession numbers. The RNA-Seq raw data files are accessible through the Gene Expression Omnibus (GEO) under accession number GSE66328. Proteomic data associated with this study can be downloaded from the ProteomeXchange under accession number PXD001924.

RESULTS

RNA-Seq and proteome profiling of *B. cenocepacia* H111 in response to nitrogen limitation. In a previous study (37), it was shown that H111 was able to utilize about 90% of the 95 nitrogen compounds available on a Biolog plate (PM3) under aerobic growth conditions. Ammonium was among the best-utilized nitrogen compounds and was chosen as a nitrogen source for growth of cells under nitrogen-replete (15 mM NH_4Cl) or nitrogen-starved (0.3 mM NH_4Cl) conditions. RNA-Seq analysis of these nitrogen-replete and -limited cells showed that 201 genes were significantly differentially regulated ($P < 0.12$) (Fig. 1). Analysis based on EggNOG protein function classification (see Materials and Methods) revealed that three categories, amino acid metabolism and transport, cell wall/membrane/envelope biogenesis, and signal transduction, were overrepresented in the 137 genes upregulated under low-nitrogen conditions. Among the genes known to be involved in nitrogen metabolism in many bacteria, we found that *glnB* (I35_2936; coding for the PII sensing protein), the glutamine synthetase gene *glnA* (I35_2151), the adjacent histidine kinase gene *ntrB* (I35_2150), *amtB* (ammonium transporter gene; I35_2935), the urease operon (I35_0766 to I35_0770; *ureECBAD* cluster), the assimilatory nitrate reductase

cluster (I35_5547 and I35_5548), and the corresponding urea and nitrate transporter genes were all upregulated under nitrogen limitation conditions (Table 1). Moreover, two large gene clusters (I35_4767 to I35_4777 [*bce*-I] [42] and I35_4922 to I35_4929 [*bce*-II] [43]) coding for the EPS cepacian, an operon encoding three lectins (I35_4182 to I35_4184; *bclBCA*) (44), and genes involved in flagellar biosynthesis (I35_3135 to I35_3139) showed significantly increased expression under nitrogen limitation conditions. Other upregulated genes encode enzymes potentially involved in PHB storage (phasin, encoded by *phaP*, and the PHB polymerase encoded by I35_4672) and the oxidative stress response (the glutathione *S*-transferase encoded by I35_4673) (Table 1). Finally, several genes encoding regulatory proteins were identified, including two conserved regulators of the nitrogen regulatory system (NtrB and NasT), two proteins with an EAL (phosphodiesterase) domain (I35_2608 and I35_3013), and a cyclic AMP-type regulator (I35_4674). Among the genes downregulated by nitrogen limitation were the pyochelin biosynthetic clusters (I35_6123 to I35_6126 and I35_6117 to I35_6121), the ECF sigma factor gene *rpoE*, and the gene for its negative regulator, *mucB* (see Table S3 in the supplemental material).

A parallel shotgun proteomic analysis of cells grown under nitrogen-starved or -replete conditions identified 2,792 proteins under starving and 2,854 proteins under replete conditions, with 2,429 proteins expressed under both conditions and 3,215 proteins identified in total (see Fig. S1A in the supplemental material). This corresponds to 46% of the predicted protein-encoding genes of *B. cenocepacia* H111. In the 200 top-regulated proteins (see Fig. S1B and Table S4), 44 proteins were also found among the top-regulated genes in the RNA-Seq analysis. From the 137 upregulated genes, 78 corresponding gene products could be detected, and among them, 58 (74%) were also found to be upregulated (fold change of $\geq$1.5) (Table 1), including the following proteins: GlnA, GlnB, the nitrogen regulatory protein NtrB, the urease α-subunit UreC, the urea transporter UrtE, the nitrate reductase and the regulator NasT, proteins of both cepacian biosynthesis clusters, the PHB depolymerase, and the granule-associate protein PhaP (Table 1). The overlap between the 137 activated genes (RNA-Seq) and the detected proteins and the results of the shotgun proteomic analysis are shown in Table 1 and in Table S4, respectively.

***In silico* search for σ^{54} binding sites.** An *in silico* DNA motif search was performed on the list of all promoters (200 nucleotides upstream of the translational start site) in the *B. cenocepacia* H111 genome (6,933 annotated genes) by using a published position-specific frequency matrix (PSFM) based on previously described σ^{54} binding sites (31). By using a *P* value threshold of 5×10^{-5}, 107 putative σ^{54} binding sites were found in the genome of H111 (1.5% of all annotated genes), and 9 of them were located upstream of the 137 genes activated under nitrogen starvation conditions (6.6%) (Table 1). By taking into account that several of these 137 activated genes are predicted to be organized in operons, these 9 binding sites may be responsible for σ^{54}-dependent transcription of 23 genes (Table 1, genes marked in bold), a significant enrichment that suggests an important role of σ^{54} in the activation of the nitrogen response. The highest-scoring σ^{54} promoters were upstream of I35_0769, coding for the urease, followed by *glnA* (I35_2151) and *glnB* (I35_2936). This is in perfect agreement with the literature, in which *glnA* and *glnB* are often used as paradigms for σ^{54}-dependent transcription (45). Other highly induced genes under nitrogen starvation conditions (Table 1) showed a significant σ^{54} binding site in their upstream promoter sequences. These included I35_0770 to I35_0775 and I35_5548, which encode the urea and nitrate transporters, respectively; I35_6432, encoding a malate symporter; I35_4672, coding for a PHB depolymerase; a cluster encoding a protein with a transglutaminase domain (I35_5080 to I35_5083); I35_6218, coding for an AraC-type transcriptional regulator; and I35_4669 and I35_6602, encoding two hypothetical proteins. With the aid of WebLogo (http://weblogo.berkeley.edu/), we used the sequences present in the 107 potential σ^{54}-dependent promoters to construct a new σ^{54} consensus sequence (see Fig. S2 in the supplemental material) with a position-specific scoring matrix (PSSM) (see Table S5) for *B. cenocepacia* H111, which will be valuable for future studies of σ^{54}-regulated gene expression in this genus.

Construction and growth analysis of a *B. cenocepacia* H111 σ^{54} mutant and a complemented derivative. In order to study the role of σ^{54} in response to nitrogen limitation, an H111 σ^{54} mutant and a complemented mutant were constructed. The growth of the *B. cenocepacia* H111 σ^{54} mutant was examined in complex LB medium or in minimal medium containing ammonium as the nitrogen source. While the mutant had a slight delay in growth in LB medium (see Fig. S3A in the supplemental material), the growth delay was more pronounced in minimal medium (see Fig. S3B). We observed that under the conditions tested, the σ^{54} mutant reached a cell density similar to that of the wild type, albeit with a delay of 6 h. The increased levels of transcripts/proteins involved in the catabolism of nitrogenous compounds (such as nitrate and urea; see above) that were observed when cells were grown under ammonium-limited conditions prompted us to test the ability of the σ^{54} mutant, the wild type, and the complemented σ^{54} mutant to utilize these nitrogen sources in minimal medium. In the presence of nitrate as the only nitrogen source, the mutant was unable to grow, while the complemented σ^{54} mutant grew to wild-type levels (Table 2). This result is consistent with results obtained for several *Pseudomonas* and *Ralstonia* strains, in which nitrate assimilation has been shown to be σ^{54} dependent (16, 46). Furthermore, other nitrogen sources, such as urea and the amino acids serine and ornithine, were used only by the wild-type strain, not the σ^{54} mutant. These phenotypic results are consistent with the data obtained in our transcriptomic analysis.

Mapping of the *B. cenocepacia* H111 σ^{54} regulon. To determine the σ^{54} regulon, RNA was extracted from cells grown first in normal minimal medium (15 mM NH_4Cl) until the exponential growth phase and then shifted to nitrogen-limited conditions (0.3 mM NH_4Cl) for 1 h. The transcriptional profiles of the *B. cenocepacia* wild-type strain H111, the isogenic σ^{54} mutant, and the complemented mutant were analyzed by RNA-Seq. This transcriptome analysis showed that 276 genes were significantly differentially expressed ($P < 0.12$) (see Fig. S4 and Table S6 in the supplemental material). The expression of 176 genes was downregulated in the σ^{54} mutant, suggesting positive control by σ^{54} (see Table S6). For the vast majority of the downregulated genes, gene expression could be restored to wild-type levels by genetic complementation (Fig. 2). Of the 176 genes downregulated in the σ^{54} mutant, 131 also showed increased expression under nitrogen starvation conditions (fold change of $\geq$1.5), providing further evidence that σ^{54} plays a central role in the control of nitrogen metabolism. Forty-six of the 176 genes activated by σ^{54} contained a putative σ^{54} consensus sequence in the promoter region, sug-

TABLE 1 Genes with statistically significantly increased expression under nitrogen starvation conditions[e]

Class	Locus ID[a]	Ortholog in J2315[b]	Description[c]	Gene name	Fold change in expression (S vs N)[d]	
					Transcript	Protein
Amino acid metabolism and transport	I35_0052	BCAL0049	Aspartate aminotransferase		33.8	16.2
	I35_0053	BCAL0051	Lysine-arginine-ornithine-binding periplasmic protein		10.8	13.1
	I35_0117	BCAL0110	Aminotransferase, DegT family		7.1	0.3
	I35_0766	**BCAL3107**	**Urease accessory protein**	***ureE***	**NA**	**6.7**
	I35_0767	**BCAL3106**	**Urease alpha subunit**	***ureC***	**14.8**	**25.1**
	I35_0770	**BCAL3103**	**Urease accessory protein**	***ureD***	**23.5**	**2.2**
	I35_0771	**BCAL3102**	**Urea ABC transporter, ATPase protein**	***urtE***	**14.6**	**17**
	I35_0772	**BCAL3101**	**Urea ABC transporter, ATPase protein**	***urtD***	**12.7**	**7.1**
	I35_0773	**BCAL3100**	**Urea ABC transporter, permease protein**	***urtC***	**14.2**	**5.2**
	I35_0774	**BCAL3099**	**Urea ABC transporter, permease protein**	***urtB***	**9.1**	**16.8**
	I35_0775	**BCAL3098**	**Urea ABC transporter, urea binding protein**		**58.9**	**59.2**
	I35_1698	BCAL1782	ABC transporter, periplasmic protein		NA	ND
	I35_2151	**BCAL2224**	**Glutamine synthetase type I**	***glnA***	**6.6**	**2.9**
	I35_2936	**BCAL0729**	**Nitrogen regulatory protein PII**	***glnB***	**14.9**	**5.8**
	I35_5082	**BCAM1235**	**Protein containing transglutaminase-like domain**		**17.9**	**23**
	I35_5083	**BCAM1236**	**Large protein containing transglutaminase-like domain**		**8.3**	**62.9**
	I35_7738	BCAS0575	Putative amino acid ABC transporter, permease protein		62.3	ND
	I35_7740	BCAS0577	ABC-type amino acid transport		40.7	39.1
	I35_7810		Aminopeptidase	*ypdF*	30.9	ND
Carbohydrate metabolism and transport	I35_4472	BCAM0577	Sialic acid transporter	*nanT*	7.5	16
Cell motility	I35_0121	BCAL0114	Flagellar biosynthesis protein	*fliC*	12.7	0.2
	I35_0148	BCAL0141	Flagellar biosynthesis protein	*flhA*	7.5	ND
	I35_3097	BCAL0567	Flagellar hook protein	*flgE*	18.3	0.2
	I35_3098	BCAL0566	Flagellar basal body rod modification protein	*flgD*	13.7	ND
	I35_3100	BCAL0564	Flagellar basal body rod protein	*flgB*	20.0	ND
	I35_3140	BCAL0525	Flagellar M-ring protein	*fliF*	11.6	0.1
Cell wall, membrane, envelope biogenesis	I35_0757	BCAL3115	Glycosyltransferase, family 2	*wbxA*	13.0	0.8
	I35_1705	BCAL1790	Ferric siderophore transport protein	*tonB*	50.3	ND
	I35_3288	BCAL3508	CidA-associated membrane protein	*cidB*	11.4	ND
	I35_4182	BCAM0184	Lectin	*bclB*	9.8	0.4
	I35_4183	BCAM0185	Lectin	*bclC*	14.9	ND
	I35_4184	BCAM0186	Lectin	*bclA*	10.4	1.2
	I35_4616	BCAM0717	Outer membrane protein (porin)		16.5	3.2
	I35_4767	BCAM0854	Mannose-1-phosphate guanylyltransferase (GDP)	*bceA*	51.6	25
	I35_4768		Undecaprenyl phosphate galactosephosphotransferase	*bceB*	31.0	2.5
	I35_4769	BCAM0855	UDP-glucose dehydrogenase	*bceC*	248.5	13.1
	I35_4771	BCAM0858	Polysaccharide export lipoprotein	*bceE*	64.1	13.1
	I35_4772	BCAM0859	Tyrosine protein kinase	*bceF*	43.5	23.6
	I35_4773	BCAM0860	Glycosyltransferase, family 2	*bceG*	19.5	3.8
	I35_4774	BCAM0861	Glycosyltransferase	*bceH*	51.8	3.4
	I35_4776	BCAM0863	Glycosyltransferase	*bceJ*	98.4	4
	I35_4777	BCAM0864	Glycosyltransferase	*bceK*	40.2	17.4
	I35_4778	BCAM0865	Permeases of the major facilitator superfamily	*bceL*	14.5	ND
	I35_4922	BCAM1003	Nucleoside-diphosphate-sugar epimerases		10.9	1.2
	I35_4923	BCAM1004	GDP-mannose 4,6-dehydratase	*gca*	106.2	4.7
	I35_4924	BCAM1005	O-antigen acetylase		45.9	ND
	I35_4927	BCAM1008	Glycosyltransferase		24.6	8.3
	I35_4928	BCAM1009	O-antigen acetylase		33.4	ND
	I35_4929	BCAM1010	UTP–glucose-1-phosphate uridylyltransferase	*gtaB*	110.4	20.4
	I35_4930	BCAM1011	O-antigen acetylase		8.9	ND
	I35_4934	BCAM1015	Outer membrane protein (porin)		9.0	1.5
	I35_5089	BCAM1241	O-antigen acetylase		29.7	ND
	I35_5188	BCAM1338	Glycosyltransferase		NA	1.4
Coenzyme metabolism	I35_5550	BCAM1687	Uroporphyrinogen-III methyltransferase		106.4	2.8
	I35_7268	BCAS0253	2-Dehydropantoate 2-reductase		14.5	2.2

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TABLE 1 (Continued)

Class	Locus ID[a]	Ortholog in J2315[b]	Description[c]	Gene name	Fold change in expression (S vs N)[d] Transcript	Protein
Energy production and conversion	I35_2136	BCAL2207	Putative dihydrolipoamide dehydrogenase		8.2	2
	I35_2138	BCAL2209	Pyruvate dehydrogenase E1 component	*aceE*	13.5	2
	I35_5545	**BCAM1683**	**Assimilatory nitrate reductase large subunit**		**7.2**	**1**
	I35_5547	**BCAM1685**	**Nitrite reductase [NAD(P)H] large subunit**	***nirB***	**12.4**	**11.2**
	I35_5712	BCAM1833	Aconitate hydratase (EC 4.2.1.3)	*acnB*	16.7	1
	I35_6014	BCAM2132	2-Aminomuconate semialdehyde dehydrogenase	*nbaE*	21.2	ND
	I35_6214	BCAM2323	Putative FMN oxidoreductase		68.0	ND
	I35_6432	**BCAM2532**	**Malate-sodium symporter**		**11.2**	**0.4**
Inorganic ion transport and metabolism	I35_2276	BCAL2352	Carbonic anhydrase		38.0	1.5
	I35_2277	BCAL2353	Sulfate permease		50.8	2.6
	I35_2935	**BCAL0730**	**Ammonium transporter**		**12.9**	**10.6**
	I35_5548	**BCAM1686**	**Nitrate/nitrite transporter**		**13.5**	**2.6**
	I35_6210	BCAM2319	Phenylpropionate dioxygenase		12.6	1.6
	I35_7455	BCAS0413	ABC-type nitrate/sulfonate/bicarbonate transporter		NA	ND
Lipid metabolism	**I35_4672**	**BCAM0774**	**Poly-beta-hydroxyalkanoate depolymerase**		**93.8**	**10.8**
	I35_5198	BCAM1347	Acyl carrier protein		16.8	ND
Nucleotide metabolism and transport	I35_0014	BCAL0012	Adenylate cyclase		15.6	1.3
	I35_6180	BCAM2289	Purine nucleoside phosphorylase		7.2	1
	I35_7647		Purine-cytosine permease and related proteins		NA	ND
Posttranslational modifications	I35_0690	BCAL3171	Molybdopterin insertion into xanthine dehydrogenase	*xdhC*	7.8	ND
	I35_4673	BCAM0775	Glutathione *S*-transferase		86.7	8.8
	I35_6232	BCAM2339	Putative protein; *S*-isoprenylcysteine methyltransferase		9.4	ND
Secondary structures	I35_2135	BCAL2206	Granule-associated protein	*phaP*	48.1	1.6
	I35_4023	BCAM0023	Acetoacetate decarboxylase	*adc*	13.0	1.9
	I35_4680	BCAM0782	Prolidase		11.8	ND
	I35_4807	BCAM0894	Poly(3-hydroxybutyrate) depolymerase		8.5	4
	I35_6004	BCAM2122	Acetaldehyde dehydrogenase		NA	ND
Transcription and signal transduction	I35_0200	BCAL0209	Histone acetyltransferase HPA2		15.3	ND
	I35_2150	**BCAL2223**	**Nitrogen regulation protein**	***ntrB***	**7.9**	**4.3**
	I35_2608	BCAL2749	EAL domain protein		7.7	3.2
	I35_3013	BCAL0652	EAL domain protein		15.6	16.5
	I35_4015	BCAM0015	Hypothetical protein		NA	1.4
	I35_4612	BCAM0714	Heavy metal response transcriptional regulator		10.9	1.3
	I35_4613	BCAM0715	Signal transduction histidine kinase		10.3	ND
	I35_4674	BCAM0776	Cyclic AMP-binding protein		34.0	0.5
	I35_4770	BCAM0857	Low-molecular-weight protein tyrosine phosphatase		69.7	ND
	I35_5338	BCAM1484	Response regulator with CheY-like receiver domain		10.9	ND
	I35_5551	BCAM1688	Response regulator NasT		11.3	4.6
	I35_6218	**BCAM2327**	**AraC-type transcriptional regulator**		**11.8**	**2.4**
Other	I35_0759		Hypothetical protein		6.9	ND
	I35_1160	BCAL1247	Hypothetical protein		12.5	0.7
	I35_1483		Hypothetical protein		NA	ND
	I35_1518		Hypothetical protein		NA	ND
	I35_2235	BCAL2308	Hypothetical protein		15.1	ND
	I35_3289	BCAL3509	Holin-like protein	*cidA*	259.5	ND
	I35_4096	BCAM0087	Hypothetical protein		26.6	ND
	I35_4131	BCAM0148	Type VI secretion protein		10.2	ND
	I35_4471	BCAM0576	Hypothetical protein		8.8	1.4
	I35_4614	BCAM0716	Hypothetical protein		29.9	4.4
	I35_4615		Hypothetical protein		9.1	ND
	I35_4617	BCAM0718	Hypothetical protein		29.4	ND
	I35_4619	BCAM0720	Conserved integral membrane protein		21.5	ND
	I35_4669	**BCAM0770**	**Hypothetical protein**		**214.1**	**1.7**
	I35_4765	BCAM0852	Hypothetical protein in rubrerythrin cluster		8.7	ND

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TABLE 1 (Continued)

Class	Locus ID[a]	Ortholog in J2315[b]	Description[c]	Gene name	Fold change in expression (S vs N)[d] Transcript	Protein
	I35_4766	BCAM0853	Transposase and inactivated derivatives		26.9	8.3
	I35_4775	BCAM0862	Unknown protein	*bceI*	35.0	ND
	I35_4817		Hypothetical protein		9.6	ND
	I35_4862	BCAM0942	Hypothetical protein		15.2	ND
	I35_4925	BCAM1006	Hypothetical protein		10.8	3.3
	I35_4926	BCAM1007	Protein involved in the export of O antigen and teichoic acid		7.3	ND
	I35_5080	**BCAM1233**	**Protein containing DUF404 and DUF407 domains**		**10.4**	**12.9**
	I35_5081	**BCAM1234**	**Protein containing DUF403 domain**		**10.7**	**22.6**
	I35_5182	BCAM1332	Hypothetical protein		17.8	ND
	I35_5345	BCAM1491	Hypothetical protein		19.4	ND
	I35_5549		Hypothetical protein		460.0	ND
	I35_5753	BCAM1927	Membrane fusion protein		18.6	ND
	I35_5959		Choline dehydrogenase		NA	ND
	I35_5976		Paraquat-inducible protein B		NA	ND
	I35_5990	BCAM2109	Nonheme chloroperoxidase		14.7	ND
	I35_6002	BCAM2120	Hypothetical protein		NA	ND
	I35_6096	BCAM2208	Xanthine/uracil/thiamine/ascorbate permease family protein		12.6	ND
	I35_6233	BCAM2340	3-(3-Hydroxyalkanoyloxy)alkanoic acid synthase	*rhlA*	13.4	1.8
	I35_6573	BCAM2679	Hypothetical protein		9.4	ND
	I35_6578	BCAM2684	GCN5-related *N*-acetyltransferase		13.6	ND
	I35_6579		Hypothetical protein		33.7	ND
	I35_6602	BCAM2717	Hypothetical protein		8.7	ND
	I35_6638	BCAM2752	Putative secreted protein		8.9	1.7
	I35_7284	BCAS0270	Urea carboxylase-related aminomethyltransferase		11.1	ND
	I35_7285	BCAS0271	Urea carboxylase-related aminomethyltransferase		19.3	1
	I35_7735	BCAS0571	Salicylate hydroxylase		38.3	ND
	I35_7736	BCAS0573	Hypothetical protein		NA	ND
	I35_7814		FAD/FMN-containing dehydrogenases		NA	ND

[a] Nomenclature is given according to the data in GenBank (accession no. HG938370, HG938371, and HG938372).
[b] Orthologs were identified as described in Materials and Methods.
[c] According to EggNOG classification.
[d] Fold change in expression for comparison of the wild-type strain grown under nitrogen-limited (S) conditions and under nitrogen-replete conditions (N). NA, not applicable because the read number or spectral count for the wild type grown under nitrogen-replete conditions was 0; ND, the protein was not detected by the shotgun proteomic approach.
[e] According to DESeq analysis ($P < 0.12$). All genes with a σ^{54} box in the promoter region are indicated in bold.

gesting direct regulation (Table 3). Among the potential direct target genes of σ^{54}, we found *glnA*, the urease gene cluster (I35_0764 to I35_0770), the urea transporter genes (I35_0771 to I35_0775), the assimilatory nitrate reductase gene (I35_5547), the nitrate transporter genes (I35_1214 and I35_5548), the PHB depolymerase gene (I35_4672), a cluster coding for a protein containing a transglutaminase domain (I35_5080 to I35_5085), and several transcriptional regulator genes (I35_1215, *ntrBC*, I35_5874, and I35_6218).

Among the σ^{54}-regulated genes (see Table S6 in the supplemental material) that did not show a significant σ^{54} consensus sequence in the promoter region with our stringent threshold, we found several genes involved in biofilm formation: genes in the two cepacian clusters, i.e., *bce*-I (I35_4771 and I35_4772) and *bce*-II (I35_4934); the large surface protein-encoding gene *bapA* (I35_6025); two lectin genes, *bclA* (I35_4184) and *bclC* (I35_4183); and *aidA*, which encodes a protein required for virulence against the nematode *Caenorhabditis elegans* (47). The σ^{54}-dependent expression of genes belonging to each of the cepacian clusters (I35_4183 and I35_4767), *bapA*, the lectin genes, and *aidA* was confirmed by real-time quantitative PCR (Table 4).

PHB production increases under nitrogen starvation conditions and is regulated by σ^{54}. Among the highly induced genes under nitrogen-limited conditions, we found genes that potentially encode functions involved in the accumulation and stability of the storage compound PHB, such as the PHB depolymerase gene (I35_4672) (48) and the phasin gene *phaP* (I35_2135) (49). Moreover, I35_4672 showed a σ^{54} consensus sequence in the promoter region and was among the genes highly regulated by σ^{54} (Table 3). In order to investigate if PHB accumulated under nitrogen starvation conditions and whether this accumulation was dependent on σ^{54}, PHB granules of the wild type, the σ^{54} mutant, and the complemented mutant grown under nitrogen-starved and nitrogen-replete conditions were examined microscopically after staining of the cells with the lipophilic dye Nile blue. When the wild-type cells were harvested from nitrogen-limited agar plates, the presence of PHB granules was readily visible and clearly increased compared to that in cells from plates containing large amounts of ammonium (Fig. 3). Whereas intracellular granules were abundant in nitrogen-limited wild-type and complemented mutant cells, these structures were significantly decreased in

TABLE 2 Nitrogen sources that were differentially used by the σ^{54} mutant[a]

Nitrogen source	Utilization		
	Wild type	σ^{54} mutant	Complemented σ^{54} mutant
15 mM NH_4Cl	+	+	+
0.3 mM NH_4Cl[b]	+	−	+
15 mM NO_3^-	+	−	+
7.5 mM urea	+	−	+
3.75 mM agmatine	+	+	+
7.5 mM L-ornithine[c]	+	−	+
15 mM L-serine[c]	+	−	+

[a] Growth was assessed by measuring the optical density at 600 nm after incubation of cells in 4 ml minimal medium supplemented with the indicated nitrogen source for 24 h at 37°C and 220 rpm. +, utilization ($OD_{600} > 0.36$); −, no utilization ($OD_{600} < 0.36$).
[b] Growth was assessed in 100 ml minimal medium (500-ml Erlenmeyer flask).
[c] Growth with L-ornithine or L-serine as the nitrogen source was assessed for 36 h.

the σ^{54} mutant, suggesting a σ^{54}-dependent regulation of PHB accumulation under nitrogen starvation conditions (Fig. 3).

EPS production, biofilm formation, and motility in *B. cenocepacia* H111 are regulated by σ^{54}. Since the expression of both the *bce*-I and *bce*-II clusters, which code for the production of the main EPS cepacian (42, 43), was positively regulated by σ^{54} and induced under nitrogen starvation conditions, EPS production was tested in a σ^{54} mutant and compared to that in the wild type by using plates containing 0.4% mannitol and 0.05% yeast extract (Fig. 4A). Visual inspection of the plates indicated that the σ^{54} mutant produced significantly reduced amounts of EPS compared to the wild type and the complemented mutant, suggesting a positive regulation of EPS production through σ^{54}. When the concentration of yeast extract in the plates was increased to 0.2%, the differences between the wild type and the mutants diminished (data not shown), suggesting that EPS production is stringently regulated via σ^{54} under conditions of nitrogen limitation.

The observation that the expression of *bapA*, coding for a large surface protein known to be important for biofilm formation on abiotic surfaces (44, 50), was activated by σ^{54} (see Table S6 in the supplemental material) prompted us to test whether biofilm formation is dependent on σ^{54}. Biofilm formation by the wild type, the σ^{54} mutant strain, and the complemented mutant was tested in a static microtiter plate assay. The results showed that the σ^{54} mutant produced approximately 60% less biofilm than the wild type and that this defect could be restored completely by complementation (Fig. 4B). Because motility is important for biofilm formation and because flagellar genes such as *motA* and *motB* (I35_4675 and I35_4676; encoding the flagellar motor) were downregulated in the σ^{54} mutant, motility assays were carried out. The σ^{54} mutant showed significantly reduced swimming and swarming motilities compared to the wild type and the complemented mutant strain (Fig. 4C and data not shown, respectively).

σ^{54} plays a role in virulence. Since expression of *aidA*, a gene required for *C. elegans* killing (47), was found to be σ^{54} dependent (Table 4; see Table S6 in the supplemental material), the importance of σ^{54} in virulence was tested in a *Caenorhabditis elegans* infection model (Fig. 5). When wild-type *B. cenocepacia* H111 was tested for pathogenicity against *C. elegans*, the large majority of the worms could develop only to the L1-L2 larval stage. In marked contrast, we observed a reduction in virulence when the assay was performed with the σ^{54} mutant, since the majority of the worms developed to the L4-adult stage. These results demonstrate that σ^{54} is involved in the pathogenicity of the bacterium against *C. elegans*, possibly through regulating *aidA* expression.

DISCUSSION

Bacteria are often confronted with an unreliable availability of nutrients in the environment and frequently have to endure limitation of nutrients, including nitrogenous compounds. In this study, the global response to nitrogen limitation in the opportunistic pathogen *B. cenocepacia* H111 was characterized, providing a data set in addition to previous transcriptomic studies done with *B. cenocepacia* J2315 grown under different stress conditions relevant to survival in the natural environment (51). As in enteric bacteria (7), the expression of key enzyme genes involved in nitrogen metabolism was found to be induced under nitrogen-limited conditions (*glnA*, coding for GS; *glnB*, encoding the nitrogen regulatory PII protein; *glnD*, coding for the bifunctional UTase/UR enzyme; and the *ntrBC* regulatory genes). However, *gdhA* transcripts, coding for the glutamate dehydrogenase, were downregulated under nitrogen-limited conditions, suggesting that nitrogen is assimilated primarily via GS/GOGAT in *B. cenocepacia* H111 in medium containing limiting amounts of ammonium. In line with this result, we showed that a *glnA-gfp* transcriptional fusion was strongly induced under ammonium limitation conditions (see Fig. S5 in the supplemental material). Interestingly, *B. cenocepacia* H111 contains two paralogs of the PII protein, but only one of the two genes (*glnB*; I35_2936) was activated by nitrogen limitation. Other nitrogen assimilation genes/proteins activated during growth in limited nitrogen were the nitrate reductase and the urease, as well as their transporters and several regulators of nitrogen metabolism (NtrBC and NasT). Accordingly, growth tests showed that the *B. cenocepacia* H111 σ^{54} mutant was unable to efficiently use nitrate or urea as a nitrogen source (Table 2). The fact that a significant number of genes activated by nitrogen starvation showed a σ^{54} box in the promoter

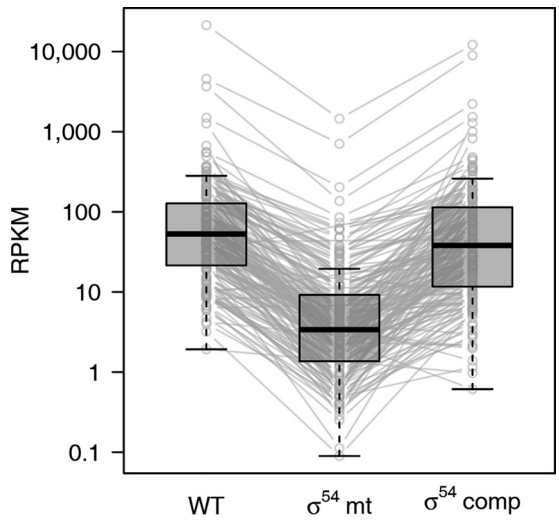

FIG 2 Box plot for the numbers of reads per kilobase per million (RPKM) for genes in the wild-type strain *B. cenocepacia* H111, the σ^{54} mutant (mt), and the complemented mutant (comp). The data show that the expression levels of the 176 genes downregulated in the σ^{54} mutant were restored to wild-type levels in the complemented mutant.

TABLE 3 Genes downregulated in the σ^{54} mutant compared to the H111 wild-type strain (DESeq $P < 0.12$) and harboring σ^{54} binding sites in their promoter regions

Locus ID[a]	Ortholog in J2315[b]	Description[c]	Gene name	Fold change in expression	
				σ^{54} mutant vs wt[d]	Complemented σ^{54} mutant vs wt[e]
I35_0068	BCAL0066	Ethanolamine operon regulatory protein		0.008	1.4
I35_0764	BCAL3109	Urease accessory protein UreG	*ureG*	0.05	0.6
I35_0765	BCAL3108	Urease accessory protein UreF	*ureF*	0.02	0.8
I35_0766	BCAL3107	Urease accessory protein UreE	*ureE*	0.02	1.1
I35_0767	BCAL3106	Urease alpha subunit	*ureC*	0.03	1.2
I35_0768	BCAL3105	Urease beta subunit	*ureB*	NA	3.0
I35_0769	BCAL3104	Urease gamma subunit	*ureA*	0.07	1.6
I35_0770	BCAL3103	Urease accessory protein UreD	*ureD*	0.03	0.7
I35_0771	BCAL3102	Urea ABC transporter, ATPase protein	*urtE*	0.02	1.1
I35_0772	BCAL3101	Urea ABC transporter, ATPase protein	*urtD*	0.03	0.7
I35_0773	BCAL3100	Urea ABC transporter, permease protein	*urtC*	0.07	0.6
I35_0774	BCAL3099	Urea ABC transporter, permease protein	*urtB*	0.02	0.9
I35_0775	BCAL3098	Urea ABC transporter, urea binding protein		0.02	1.3
I35_1214	BCAL1319	Nitrite transporter		0.12	1.1
I35_1215	BCAL1320	Regulator of cell morphogenesis and NO signaling		NA	1.2
I35_1420	BCAL1523	PE_PGRS family protein		0.05	0.5
I35_1421	BCAL1524	PE_PGRS family protein		0.02	0.3
I35_1734	BCAL1818	Zn-dependent hydrolases, including glyoxylases		0.04	1.0
I35_1735	BCAL1819	Oxidoreductase (flavoprotein)		0.005	0.7
I35_1736		Oxidoreductase (flavoprotein)		0.002	0.5
I35_2149	BCAL2222	Nitrogen regulation protein NR(I)	*ntrC*	0.09	1.0
I35_2150	BCAL2223	Nitrogen regulation protein NR(II)	*ntrB*	0.07	1.3
I35_2151	BCAL2224	Glutamine synthetase type I	*glnA*	0.10	2.0
I35_2727	BCAL0940	Membrane carboxypeptidase		0.16	0.8
I35_2935	BCAL0730	Ammonium transporter	*amtB*	0.02	1.3
I35_4051	BCAM0050a	Hypothetical protein		0.10	1.4
I35_4450	BCAM0555	Alkanesulfonate-binding protein		NA	1.2
I35_4452	BCAM0557	Alkanesulfonate monooxygenase		0.04	2.3
I35_4669	BCAM0770	Hypothetical protein		0.02	4.2
I35_4672	BCAM0774	Poly-beta-hydroxyalkanoate depolymerase		0.004	0.7
I35_5080	BCAM1233	Protein containing DUF404 and DUF407 domains		0.01	1.7
I35_5081	BCAM1234	Protein containing DUF403 domain		0.004	1.0
I35_5082	BCAM1235	Protein containing transglutaminase-like domain		0.002	2.2
I35_5083	BCAM1236	Large protein containing transglutaminase-like domain		0.01	0.9
I35_5084	BCAM1237	Protein containing DUF404, DUF407, and DUF403 domains		0.11	0.8
I35_5085	BCAM1238	Protein containing transglutaminase-like domain		0.12	0.6
I35_5335	BCAM1481	Hypothetical protein		NA	0.1
I35_5545	BCAM1683	Assimilatory nitrate reductase large subunit		0.01	3.9
I35_5546	BCAM1684	Nitrite reductase [NAD(P)H] small subunit		NA	4.4
I35_5547	BCAM1685	Nitrite reductase [NAD(P)H] large subunit	*nirB*	0.04	5.2
I35_5548	BCAM1686	Nitrate/nitrite transporter		0.01	4.1
I35_5874	BCAM2039	Two-component response regulator		0.02	0.3
I35_6218	BCAM2327	Transcriptional regulator		0.04	1.3
I35_7023	BCAS0024	Transcriptional regulator GabR of GABA utilization		0.08	1.6
I35_7622		D-Glycero-D-manno-heptose 1,7-bisphosphate phosphatase		0.07	1.3
I35_7626		Conserved domain protein		0.06	1.6

[a] Nomenclature is given according to data in GenBank (accession no. HG938370, HG938371, and HG938372).
[b] Orthologs were identified as described in Materials and Methods.
[c] According to EggNOG classification.
[d] Fold change in transcript expression for comparison of the σ^{54} mutant and the wild type (wt) grown under nitrogen-limited conditions. NA, not applicable because the read number for the mutant strain was 0.
[e] Fold change in transcript expression for comparison of the complemented σ^{54} mutant and the wild type (wt) grown under nitrogen-limited conditions.

region suggested that in *B. cenocepacia* H111, this alternative sigma factor plays an important role in controlling nitrogen assimilation. This was confirmed by RNA-Seq analysis of a σ^{54} mutant strain grown under nitrogen-limited conditions, as the set of genes activated by σ^{54} overlapped, to a large extent (74%), the genes upregulated under nitrogen limitation.

In addition to genes involved in nitrogen metabolism, other genes, coding for diverse functions, were activated by low nitrogen in a σ^{54}-dependent manner. For example, we noticed that the expression of genes involved in PHB accumulation and stability (a PHB depolymerase gene and the phasin *phaP* gene, whose products bind to PHB and promote PHB synthesis [49]) was strongly

TABLE 4 qPCR results for selected genes

Locus ID[a]	J2315 ortholog[b]	Description[a]	Fold change for mutant vs wt[c]
I35_6025	BCAM2143	Large protein BapA	−1.8 ± 0.2
I35_4183	BCAM0185	Lectin BclC	−2.7 ± 0.2
I35_7309	BCAS0293	Nematocidal protein AidA	−3.2 ± 0.2
I35_4767	BCAM0854	Glycosyltransferase	−5.6 ± 0.5
I35_4923	BCAM1004	GDP-mannose 4,6-dehydratase	−4.8 ± 0.2
I35_0767	BCAL3106	Urease subunit	−84.4 ± 30.1
I35_2935	BCAL0730	Ammonium transporter	−1,277 ± 200

[a] Nomenclature and descriptions are given according to data in GenBank (accession no. HG938370, HG938371, and HG938372).
[b] Orthologs were identified as described in Materials and Methods.
[c] Fold change in qPCR expression for comparison of the σ^{54} mutant and the wild type (wt) grown in minimal medium in a shift experiment.

upregulated under nitrogen limitation conditions (Table 1). Our data also suggest that the accumulation of this carbon and energy storage compound is dependent upon the presence of a functional σ^{54}. Microscopic inspection revealed that a reduced number of PHB granules accumulated in the σ^{54} mutant compared to the wild type and the complemented mutant (Fig. 3).

The *bce*-I and *bce*-II clusters, which direct the biosynthesis of the most common type of EPS produced by *Burkholderia*, cepacian, were among the genes and proteins that were most strongly activated by low nitrogen in a σ^{54}-dependent fashion (Tables 1 and 3). Cepacian has been identified in different species, including both clinical and environmental *Bcc* isolates (43). It is composed of a branched acetylated heptasaccharide repeating unit with D-glucose, D-rhamnose, D-mannose, D-galactose, and D-glucuronic acid, at a ratio of 1:1:1:3:1 (52). Cepacian has been shown to protect cells from external stresses, such as desiccation and metal ion stress (43), and also to be involved in pathogenic interactions by increasing adherence (53) and antibiotic resistance (54, 55) and interfering with the innate immune system (54, 56). To our knowledge, we show here for the first time that cepacian production is stimulated in a σ^{54}-dependent manner under nitrogen-limited conditions (Fig. 4A). In other bacteria, EPS molecules are known to be produced in response to a nutritional stress such as nitrogen limitation (57–59); in *Vibrio fischeri* and *Vibrio vulnificus*, genes involved in the biosynthesis of EPS, which promote symbiotic colonization of their hosts, have been shown to be regulated by σ^{54} (60, 61). However, the knowledge of how EPS biosynthesis is regulated at the molecular level is still very limited. Although our stringent search for σ^{54} consensus sequences did not identify potential binding sites in the promoter regions of the cepacian clusters, a more permissive search (*P* value threshold of 0.0002) identified potential σ^{54} boxes in front of I35_4771 (*bceE*) and I35_4776 (*bceJ*), in the first Bce cluster, and in front of I35_4927 and I35_4934, in the second cluster. More in-depth molecular studies will be required to confirm that these boxes are in fact recognized by σ^{54}.

Others functions found to be controlled by σ^{54} in *B. cenocepacia* H111 were motility and biofilm formation (Fig. 4). An involvement of σ^{54} in these phenotypes was previously reported by Valvano and colleagues, who demonstrated that in *B. cenocepacia* K56-2, a functional σ^{54} protein is required for motility and biofilm formation *in vitro* (3). In contrast to other bacteria, a K56-2 σ^{54} mutant still displayed flagella on its surface, indicating that σ^{54} is not involved in flagellum biosynthesis (3). Using RNA-Seq analysis, we showed here that in *B. cenocepacia* H111, the expression of genes encoding the motor for flagellar movement (*motA* and *motB*) is regulated by σ^{54}. This is in agreement with the results obtained for K56-2, where a *motA* mutant was shown to be non-

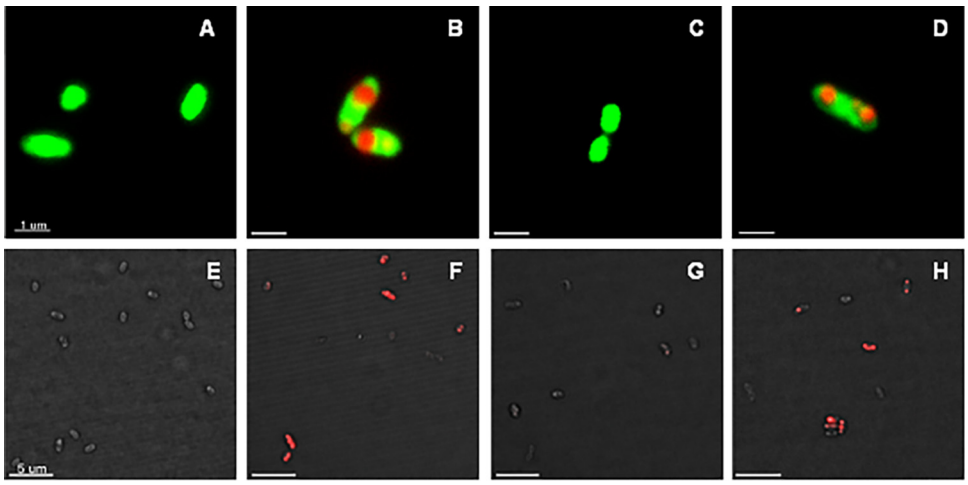

FIG 3 PHB accumulation is induced under nitrogen limitation conditions and is dependent on σ^{54}. Cells of the wild-type strain *B. cenocepacia* H111 (B and F), the σ^{54} mutant (C and G), and the complemented mutant (D and H) were grown on plates containing limiting amounts of ammonium. (A and E) As a control, the wild-type strain was also grown under nitrogen-replete conditions. The upper row shows cells stained with Syto 9 and Nile blue, whereas the lower row shows a combination of bright-field microscopy and Nile blue staining. Bars, 1 μm (A to D) and 5 μm (E to H).

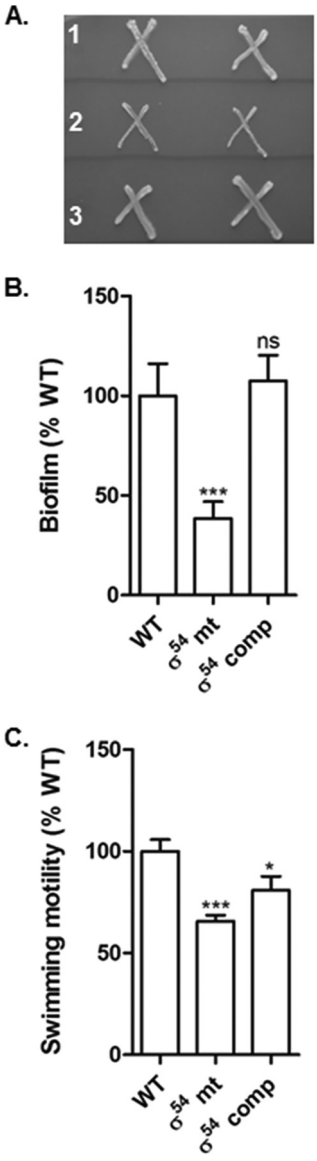

FIG 4 σ^{54}-dependent EPS production, biofilm formation, and motility. (A) EPS production was tested on mannitol medium containing 0.02% yeast extract. Row 1, wild type (WT); row 2, σ^{54} mutant; row 3, complemented mutant. (B) Biofilm formation in AB minimal medium containing citrate as the C source. (C) Swimming motility of the wild-type strain *B. cenocepacia* H111, the σ^{54} mutant, and the complemented mutant. Error bars indicate standard deviations (SD) ($n \geq 3$). Asterisks indicate a significant difference from the wild-type strain (***, $P < 0.001$; *, $P < 0.5$; unpaired two-tailed *t* test). ns, not significant.

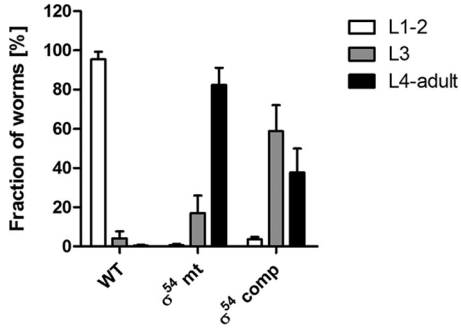

FIG 5 Virulence of *B. cenocepacia* H111 against *C. elegans* is dependent on σ^{54}. The σ^{54} mutant showed reduced virulence compared with the parental wild-type strain or the complemented mutant strain in a *C. elegans* infection model. Error bars indicate standard errors of the means (SEM) ($n = 4$).

motile but produced intact flagella (3). Our data suggest that the drastic reduction in biofilm formation of an H111 σ^{54} mutant is most likely due to decreased expression of *bapA*, which encodes the large surface protein BapA (fold change of 0.15) (see Table S6 and Fig. S6 in the supplemental material). A *bapA* mutant has previously been shown to be defective in biofilm formation (44). We also observed a reduction in *C. elegans* pathogenicity when σ^{54} was inactivated. This reduction was due, at least in part, to lower levels of *aidA* transcripts (47) in the σ^{54} mutant strain (fold change of 0.1) (see Table S6). We observed that the pathogenicity toward *C. elegans* was only partially restored in the complemented σ^{54} mutant. Since the expression of σ^{54} in the complemented mutant was driven by the promoter of the expression vector, not by its natural promoter, it is possible that some fine-tuning of expression (i.e., temporal) is missing and, as a result, pathogenicity is not fully restored to the level of the wild type.

We were unable to identify a σ^{54}-dependent promoter upstream of *aidA*, suggesting an indirect regulation of this gene by σ^{54}. In contrast, when we used a more permissive *P* value threshold, a potential σ^{54} binding site was found upstream of *bapA* ($P = 0.0016$) (TGGTTGAACGTTTGCC), suggesting a potential direct regulation and adding an extra layer of complexity to the regulation of this gene, which has been shown to be regulated by two quorum sensing molecules, *N*-octanoyl homoserine lactone (C_8-HSL) and BDSF (*Burkholderia* diffusible signal factor; *cis*-2-dodecenoic acid) (62).

Our results reveal that σ^{54} may be an important factor in the metabolic adaptation of *B. cenocepacia* H111 to stressful and nutrient-limited environments, such as the CF lung. Recent studies using immunohistochemistry visualized the spatial distribution of *Bcc* members in the lungs of CF patients (63, 64). In these studies, *Bcc* bacteria were shown to grow as either single cells or small clusters within macrophages or mucus. Very relevant to these observations and to our present study is the fact that for another *B. cenocepacia* strain (K56-2), σ^{54} was shown to be important for the intracellular trafficking and survival of this clinical isolate of *B. cenocepacia* within infected macrophages (3). Our findings may give an explanation for how *Bcc* bacteria adapt to and thrive in the CF lung. Additional work will be required to unravel the precise molecular mechanisms underlying the activation of σ^{54}-dependent candidate genes and to identify the activator proteins interacting with this alternative sigma factor.

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