







Reclassification of the Specialized Metabolite Producer *Pseudomonas mesoacidophila* ATCC 31433 as a Member of the *Burkholderia cepacia* Complex

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ABSTRACT *Pseudomonas mesoacidophila* ATCC 31433 is a Gram-negative bacterium, first isolated from Japanese soil samples, that produces the monobactam isosulfazecin and the β -lactam-potentiating bulgecins. To characterize the biosynthetic potential of *P. mesoacidophila* ATCC 31433, its complete genome was determined using single-molecule real-time DNA sequence analysis. The 7.8-Mb genome comprised four replicons, three chromosomal (each encoding rRNA) and one plasmid. Phylogenetic analysis demonstrated that *P. mesoacidophila* ATCC 31433 was misclassified at the time of its deposition and is a member of the *Burkholderia cepacia* complex, most closely related to *Burkholderia ubonensis*. The sequenced genome shows considerable additional biosynthetic potential; known gene clusters for malleilactone, ornibactin, isosulfazecin, alkyhydroxyquinoline, and pyrrolnitrin biosynthesis and several uncharacterized biosynthetic gene clusters for polyketides, nonribosomal peptides, and other metabolites were identified. Furthermore, *P. mesoacidophila* ATCC 31433 harbors many genes associated with environmental resilience and antibiotic resistance and was resistant to a range of antibiotics and metal ions. In summary, this bioactive strain should be designated *B. cepacia* complex strain ATCC 31433, pending further detailed taxonomic characterization.

IMPORTANCE This work reports the complete genome sequence of *Pseudomonas mesoacidophila* ATCC 31433, a known producer of bioactive compounds. Large numbers of both known and novel biosynthetic gene clusters were identified, indicating that *P. mesoacidophila* ATCC 31433 is an untapped resource for discovery of novel bioactive compounds. Phylogenetic analysis demonstrated that *P. mesoacidophila* ATCC 31433 is in fact a member of the *Burkholderia cepacia* complex, most closely related to the species *Burkholderia ubonensis*. Further investigation of the classification and biosynthetic potential of *P. mesoacidophila* ATCC 31433 is warranted.

KEYWORDS genome, identification, antibiotic resistance, metal resistance, antibacterial, biosynthesis, bulgecin

Pseudomonas mesoacidophila was isolated from Japanese soil samples in the late 1970s (1). It is known to produce the sulfamate monobactam isosulfazecin (1) and the bulgecins (2), a group of sulfated glycopeptides that inhibit lytic transglycosylases

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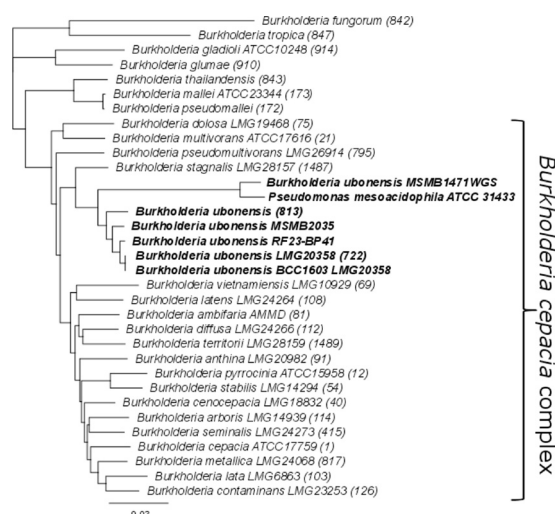


FIG 1 Phylogeny of *P. mesoacidophila* ATCC 31433 within the genus *Burkholderia*. The analysis was based on the sequences of six MLST alleles (*trpB*, *recA*, *lepA*, *gyrB*, *gltB*, and *atpD*). The PubMLST isolate identification numbers are shown in parentheses after the species names.

(3) and metallo- β -lactamases (4) and potentiate β -lactam activity (2, 5). *Pseudomonas* and other soil Gram-negative bacterial genera, such as *Stenotrophomonas* and *Burkholderia*, often show high levels of resistance to antibiotics (6). For example, *Pseudomonas aeruginosa* is a common nosocomial pathogen with reduced susceptibility to a range of antibiotics, due to low membrane permeability, chromosomal multidrug efflux pumps, overexpressed β -lactamases, and the acquisition of multidrug resistance plasmids (6, 7). *Stenotrophomonas maltophilia* is an emerging nosocomial pathogen (8), and several *Burkholderia* species, even environmental isolates, are resistant to a wide range of antibiotic classes (9). Species of both *Pseudomonas* and *Burkholderia* produce an array of specialized bioactive metabolites (10, 11). Despite these findings, the full biosynthetic potential and antibiotic susceptibility of *P. mesoacidophila* have not been reported. Here, we report the complete genome sequence of *P. mesoacidophila*, and we show that this species shares an antimicrobial-resistant phenotype characteristic of *Burkholderia* and should be reclassified phylogenetically as a member of the *Burkholderia cepacia* complex.

RESULTS

Genome sequencing and taxonomic placement of *P. mesoacidophila* ATCC 31433 within the *Burkholderia* genus. Previous studies showed *P. mesoacidophila* ATCC 31433 to be a producer of specialized bioactive metabolites (1, 2, 12). There is only one known example of this species, with no nucleotide sequences reported. Amplification and sequencing of *16S*, *recA*, and *gyrB* genes indicated that these sequences were closely related to those of *Burkholderia* species (data not shown) (13). To characterize this isolate, we obtained a complete genome sequence using PacBio technology. The sequence assembled into four contigs, with a total size of 7.84 MB. The sequences of six multilocus sequence typing (MLST) loci from the genome were compared to those of characterized *Burkholderia* species. *P. mesoacidophila* ATCC 31433 clustered most closely with *Burkholderia ubonensis* within the *Burkholderia cepacia* complex (Fig. 1), which confirms its misclassification.

The genome sequence of *P. mesoacidophila* ATCC 31433 was compared to that of *B. ubonensis* MSMB1471 (GenBank accession numbers [NZ_CP013462](#), [NZ_CP013463](#), [NZ_CP013464](#), and [NZ_CP013465](#)) (Fig. 2). Both genomes were organized similarly in three chromosomes, each encoding rRNA, and were largely syntenic. The *B. ubonensis* MSMB1471 and *P. mesoacidophila* ATCC 31433 genomes shared an average nucleotide identity (ANI) of 94.7%, which falls below the proposed species-level cutoff value of

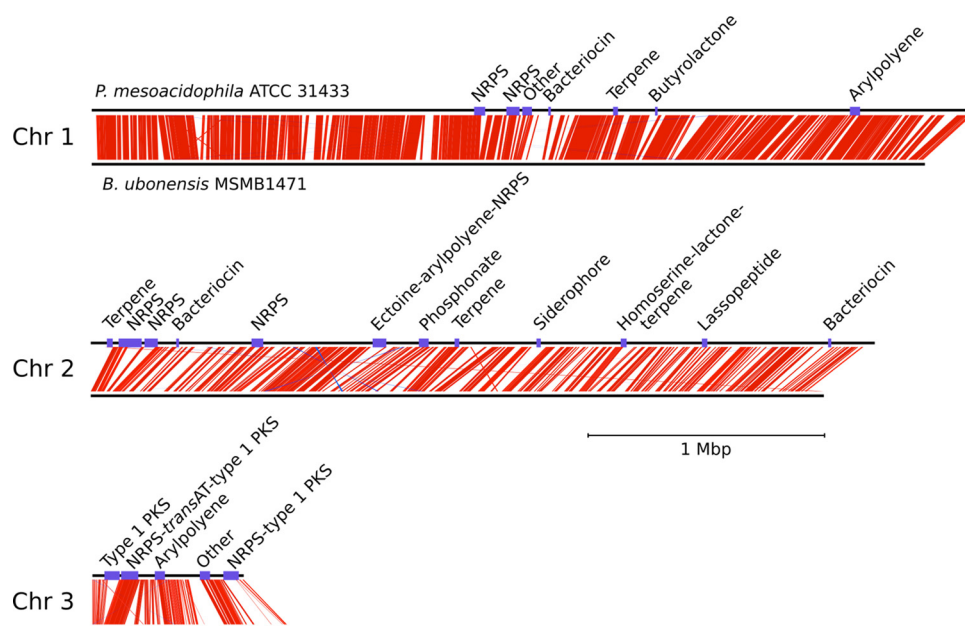


FIG 2 Genome sequence comparison of *P. mesoacidophila* ATCC 31433 and *B. ubonensis* MSMB1471. Each chromosome (Chr) is displayed separately and to scale (as indicated), with *P. mesoacidophila* ATCC 31433 above and *B. ubonensis* MSMB1471 below. Regions of homologous sequences are linked with red lines. AntiSMASH-predicted gene clusters are shown in purple. The smallest contig of the *P. mesoacidophila* ATCC 31433 genome is not shown, because it neither had homology with *B. ubonensis* MSMB1471 nor contained any antiSMASH-predicted biosynthetic pathways.

95% (14). These data suggest that *P. mesoacidophila* ATCC 31433, while clearly a member of the *B. cepacia* complex and related to *B. ubonensis*, may yet be a novel species within this taxonomic subgroup of *Burkholderia*.

General features of the *P. mesoacidophila* ATCC 31433 genome. Annotation of the genome revealed numerous elements that suggest resilience to environmental conditions. Genes are present encoding detoxification proteins, such as nitric oxide dioxygenase (flavo-hemoglobin), superoxide dismutase, catalase, catalase/peroxidase, thiol peroxidase, alkylhydroperoxidase, alkylhydroperoxide reductase, alkylsulfonate monooxygenase, and transport proteins, and a range of proteins involved in sulfite metabolism, DNA repair, and the osmotic stress response. Genes and gene clusters required for uptake and metabolism of chitin, collagen, pectin, phenylpropanoids, phospholipids, glycerol, arabinose, ribose, fucose, xylose, trehalose, polyols, glycolate, lactate, 2-ketogluconate, hydantoin, allantoin, heme, taurine, putrescine, and methylamine were all identified. Siderophore synthetases, transport proteins, and receptors are present, along with several other proteins involved in metal homeostasis. Genes encoding a wide range of antibiotic resistance proteins and efflux pumps are also present.

Interactions with the environment are also mediated by the chaperone-usher fimbriae, Flp pili, type IV pili, adhesins, and biofilm-forming proteins (PgaABCD and PelABCDEFG) identified, while motility is suggested by genes encoding flagellar components. Lipopolysaccharide biosynthesis genes include those for O-antigen biosynthesis and lipid A modification. A number of genes and gene clusters involved in specialized metabolite biosynthesis were indicated. In addition, the genome contains several phage proteins, transposases, and Tra and Trb conjugative transfer proteins.

P. mesoacidophila harbors genes encoding secreted enzymes and toxins involved in virulence and interactions with hosts, such as phospholipases (including a phosphatidylinositol-specific phospholipase C seen in other *Burkholderia* species [15]), secreted collagenase, LasA protease, secreted zinc metalloprotease, pectin-degrading polygalacturonase, HlgAB, RelBE, YgiTU, and VapBC antitoxin-toxin pairs, RTX toxin, Phd

antitoxin, and toxin secretion proteins. Components of possible type I, II, III, IV, and VI secretion systems have also been identified.

Specialized metabolite production by *P. mesoacidophila* ATCC 31433. To uncover the secondary metabolite potential of *P. mesoacidophila*, we analyzed the genome using the antiSMASH tool (16) and performed comparative investigations with other *B. ubonensis* genomes. This identified 91 putative biosynthetic gene clusters from five isolates. *P. mesoacidophila* yielded 24 clusters, suggesting 40% more biosynthetic potential than the other *B. ubonensis* isolates analyzed (Table 1).

A small proportion of the biosynthetic clusters identified are required for the biosynthesis of known compounds. Of the nine polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) clusters, three are known, three have equivalents in other *Burkholderia* species, and three have no established equivalents indicated by antiSMASH. The complete PKS cluster for malleilactone production (17) and the NRPS cluster for ornibactin biosynthesis (18) were found, and the NRPS part of the ectoine-arylpolyyne-NRPS cluster is similar to the sulfazecin biosynthesis cluster in *Pseudomonas acidophila* (19), with the exception that the SulM equivalent in *P. mesoacidophila* has no epimerization domain. Therefore, this group of genes is likely to be responsible for isosulfazecin biosynthesis. The complete four-gene cluster required for biosynthesis of the antifungal pyrrol-nitrin (20) is present, although we could not detect production of this metabolite in either culture broth or partially purified cell extracts with liquid chromatography-mass spectrometry (LC-MS). Genes necessary for the synthesis of alkylhydroxyquinolines, which are involved in quorum sensing (21), were found in one of the NRPS clusters.

One of the three terpene clusters constitutes part of the hopanoid biosynthetic pathway described for *Burkholderia cenocepacia* J2315 (encoding seven proteins with >75% identity to their counterparts HpnCEFGMN and VacJ, plus two proteins not found in that species) (15, 22). A response regulator gene just downstream of the *hpnCEFGMN* cluster encodes a protein 84% identical to Bp1026b_I12523 from *Burkholderia pseudomallei*, which is involved in thermoregulation and biofilm formation (23). Clusters equivalent (>75% identical at the protein level) to *hpnABH/ispH* and *hpnIJKL/ompA* in *B. cenocepacia* J2315 (22) were also found, making up the entire hopanoid biosynthetic pathway. The other two terpene clusters and the homoserine lactone-terpene cluster contain one isolated squalene-hopene cyclase gene and two isolated squalene synthase genes. The homoserine lactone synthase and its associated regulator found in the homoserine lactone-terpene cluster, which are involved in quorum sensing, encode proteins >85% identical to their equivalents in *B. cenocepacia* J2315 (15, 24).

In addition to core genes encoding fatty acid synthase components >80% identical to those seen in other *Burkholderia* species (25), two genes encoding cyclopropane fatty acyl phospholipid synthases (known components of *Burkholderia* cell membranes [26]) and one encoding diffusible factor synthase (90% identical at the protein level to BCAM0581 from *B. cenocepacia* J2315), which is responsible for production of a fatty acid involved in quorum sensing (27), were found. *P. mesoacidophila* also produces poly(β -hydroxybutanoate) (1), and poly(β -hydroxyalkanoate) synthase and depolymerase genes were identified.

Production of bulgecin by *P. mesoacidophila*. We sought to confirm the presence of bulgecin in the culture broth of *P. mesoacidophila* ATCC 31433. Bulgecin was shown previously to potentiate the activity of β -lactam antibiotics and to cause bulge formation in *Escherichia coli* (2, 28). After 78 h of growth at 28°C in PF medium, *P. mesoacidophila* gave 36 g of cell pellet per liter of culture and reduced the pH of the medium from 7.0 to 5.5 ± 0.5 . The medium acquired a distinctive fruity smell and remained translucent even after repeated centrifugation at $6,000 \times g$ to remove cells. The culture filtrate of *P. mesoacidophila* potentiated the lytic activity of cefuroxime against *E. coli* and caused bulge formation (Fig. 3), as expected if bulgecin were present (2, 28). *E. coli* JM109 cells exposed to a sublethal concentration of cefuroxime grew as long cell chains, consistent with this compound's selective inhibition of PBP3 and prevention of

TABLE 1 Biosynthetic gene clusters from *P. mesoacidophila* and *B. ubonensis*

Strain	No. of predicted clusters ^a															Total
	PKS		Bacteriocin-			Homoserine			Ecto- anypolyene-			Other				
	NRPS	containing ^b	Terpene	Bacteriocin	proteusin	Arylpolyene	Siderophore	Lasso-peptide	lactone- terpene	Phosphonate	Butyrolactone		NRPS			
<i>B. ubonensis</i> RF23-BP41	3	2	3	1	1	3	0	0	1	1	1	0	1	17		
<i>B. ubonensis</i> SMB1471WGS	2	3	3	1	0	4	0	0	1	1	0	0	2	18		
<i>B. ubonensis</i> MSMB2035	3	1	3	1	0	2	0	0	1	1	1	0	1	14		
<i>B. ubonensis</i> BCC 1603	4	2	3	1	0	3	0	0	1	1	1	0	2	18		
<i>P. mesoacidophila</i> ATCC 31433	5	3	3	3	0	2	1	1	1	1	1	1	2	24		
Total														91		

^aThe numbers of secondary metabolite clusters in *P. mesoacidophila* and four closely related isolates of *B. ubonensis*, as predicted by antiSMASH, are shown.
^bPKS-containing clusters include T1PKS, *trans*-AT PKS, other KS, and any mixed cluster containing one of these types.

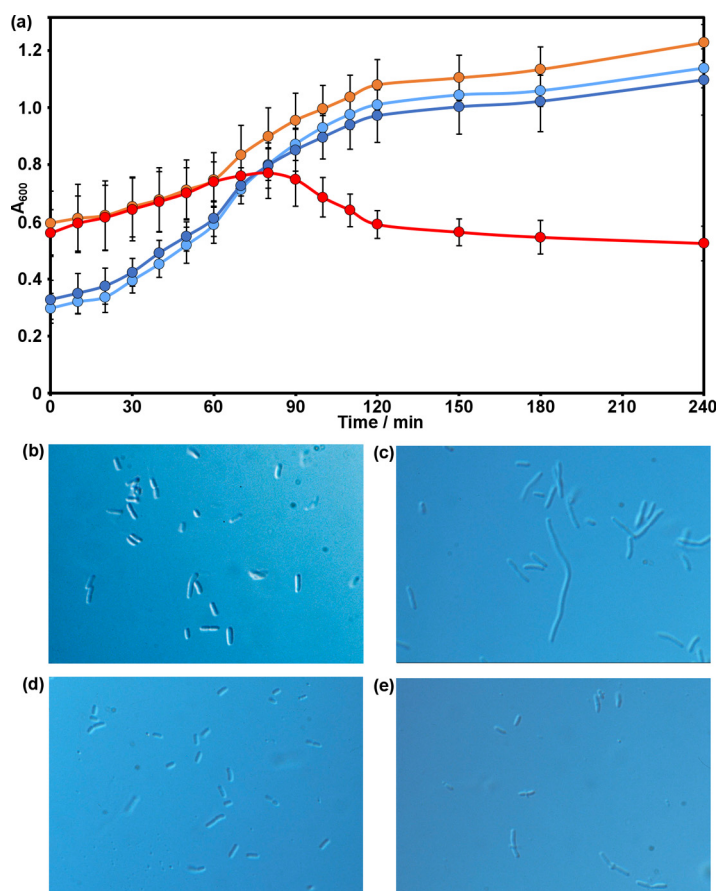


FIG 3 Cefuroxime potentiation and bulge-forming activity. (a) Growth curves of *E. coli* JM109 in PS medium without (light blue) and with (dark blue) 5 $\mu\text{g ml}^{-1}$ cefuroxime and in *P. mesoacidophila* culture filtrate without (orange) and with (red) 5 $\mu\text{g ml}^{-1}$ cefuroxime. (b) to (e) Light microscopy of *E. coli* JM109 alone (b) and exposed to 5 $\mu\text{g ml}^{-1}$ cefuroxime (c), *P. mesoacidophila* culture filtrate (d), and a combination of 5 $\mu\text{g ml}^{-1}$ cefuroxime and *P. mesoacidophila* culture filtrate (e). Cefuroxime potentiation and bulge formation are consistent with the presence of bulgecin (2, 28).

proper septation (29), whereas cells exposed to the same concentration of cefuroxime in *P. mesoacidophila* culture broth developed bulges at the sites of septation (2, 28).

Antibiotic and metal resistance of *P. mesoacidophila*. *P. mesoacidophila* was resistant to a wide range of antibiotics (Table 2), including the antipseudomonal aminoglycoside tobramycin, the carbapenems, the monobactam aztreonam, and polymyxin B. Of the 24 antibiotics tested, only the antipseudomonal β -lactams piperacillin and ceftazidime showed clear activity against *P. mesoacidophila*, with the fluoroquinolone ciprofloxacin and the fourth-generation cephalosporin cefepime showing intermediate activity. In a few cases, the zone diameters in disc diffusion assays were close to the breakpoint value (with some replicates indicating resistance and others suggesting a degree of susceptibility), while the broth microdilution method clearly indicated resistance. *Burkholderia* species are known to give poor correlation between the two methods (30). *E. coli* JM109, which was tested simultaneously, was susceptible to all antibiotics assayed except erythromycin and nalidixic acid, as expected (data not shown).

P. mesoacidophila has predicted genes for β -lactamases from classes A and C, along with other proteins containing β -lactamase domains. Inducible β -lactamase activity was demonstrated using cefoxitin (Table 3), whereas no inducible β -lactamase activity and much greater susceptibility to cefoxitin were seen for *E. coli* JM109. Two of the genes thought to encode class A β -lactamases have 63% and 54% identity with the

TABLE 2 Antibiotic susceptibility of *P. mesoacidophila* ATCC 31433, determined by the broth microdilution and disc diffusion methods

Antibiotic	Microdilution method			Disc diffusion method				Resistance
	MIC ^a (μg ml ⁻¹)	Breakpoint ^b		Dose (μg)	Zone of inhibition (mm)	Breakpoint ^b		
		R (>)	S (≤)			R (≤)	S (≥)	
Amoxicillin	>128	(8)	(8)	30	0	(14)	(15)	R
Co-amoxiclav	>128	(8)	(8)	20/10	0	(20)	(21)	R
Ampicillin	ND			25	0	(14)	(15)	R
Carbenicillin	>128			100	12	12	13	R
Piperacillin	8	16	16	75	37	24	25	S
Cefuroxime	>128	(8)	(8)	30	21	(19)	(20)	R
Cefoxitin	>128	(8)	(8)	30	8	(22)	(23)	R
Cefotaxime	16	(2)	(1)	30	23	26	27	R
Ceftazidime	8	8	8	30	29	23	24	S
Cefepime	4	(4)	(1)	30	31	(26)	(30)	I
Imipenem	ND	8	4	10	12	16	23	R
Meropenem	16	8	2	10	19	15	20	R
Aztreonam	128	16	1	30	18	19	36	R
Erythromycin	>128			30	0			R
Tetracycline	64			10	0	(23)	(24)	R
Chloramphenicol	32	(8)	(8)	30	17	(20)	(21)	R
Streptomycin	ND			10	0			R
Kanamycin	64			30	18			R
Gentamicin	128	4	4	10	9	17	18	R
Tobramycin	ND	4	4	10	11	19	20	R
Nalidixic acid	32	(16)	(16)	30	20	(17)	(18)	R
Ciprofloxacin	0.5	1	0.5	5	26	19	30	I
Rifampin	16			30	18			
Polymyxin B	64	4	4	25	14			R

^aND, not determined.^bBreakpoints are for *Pseudomonas* spp.; breakpoints in parentheses are for *Enterobacteriaceae*. The polymyxin B breakpoint is for colistin (polymyxin E). R, resistant; I, intermediate; S, susceptible.

genes encoding the known inducible class A β -lactamase from *Burkholderia mallei* and *B. pseudomallei* (31).

Resistance to polymyxin B is rare among Gram-negative species, including pseudomonads, but is well known among *Burkholderia* species, in which the modification of lipid A prevents antibiotic binding (32). Indeed, the Arn gene cluster, which is responsible for the biosynthesis of 4-amino-4-deoxyarabinose and its incorporation into lipid A (33, 34), has been identified. This outer membrane modification also confers decreased susceptibility to aminoglycosides, which, together with a predicted aminoglycoside phosphotransferase (also seen in *B. cenocepacia* strain J2315 [15]) and several membrane efflux pumps, accounts for the marked resistance to this class of antibiotics (35, 36).

The genome also carries genes encoding CreBCD colicin resistance proteins, a bleomycin (glycopeptide) resistance protein (63% identical to the Tn5-mediated bleomycin resistance protein [37], including conservation of the bleomycin-binding regions), phosphinothricin *N*-acetyltransferase (36% identical to phosphinothricin *N*-acetyltransferase from *Brucella ovis* [GenBank accession number WP_006155257; PDB accession number 5DWN], with significant conservation of the active site), various acyltransferases and glycosyltransferases that may play roles in resistance, and an undecaprenyl diphosphatase associated with bacitracin resistance (38). Genes poten-

TABLE 3 Induction of β -lactamase activity in *P. mesoacidophila* ATCC 31433 by cefoxitin

Cefoxitin concentration ($\mu\text{g ml}^{-1}$) ^a	Nitrocefin hydrolysis rate (nM s ⁻¹)	Fold induction
0	22.7 \pm 3.4	1
1	28.7 \pm 1.4	1.3 \pm 0.2
10	68.5 \pm 1.2	3.0 \pm 0.5
100	300.8 \pm 29.9	13.2 \pm 2.4

^aThe growth of *P. mesoacidophila* ATCC 31433 was unaffected by cefoxitin at the concentrations used.

TABLE 4 Metal ion susceptibility of *P. mesoacidophila* ATCC 31433, as determined by the broth microdilution method

Salt ^a	MIC (mg ml ⁻¹)	MIC (mM)
NaCl	>15	>257
KCl	>10	>133
MgCl ₂	>10	>105
CaCl ₂	>10	>90
MnCl ₂ ·4H ₂ O	5	25
FeCl ₃	1	6
CoCl ₂ ·6H ₂ O	0.25	1
NiCl ₂	1.25	10
CuCl ₂ ·2H ₂ O	1	6
ZnCl ₂	1	7
HgCl ₂	0.008	0.03

^aAll broths contained 5 mg ml⁻¹ NaCl.

tially encoding putative trimethoprim-resistant dihydrofolate reductase FolM (25% identity with FolM from *E. coli*) and chloramphenicol phosphotransferase (27% identical to the N-terminal two-thirds of chloramphenicol phosphotransferase from *Streptomyces venezuelae* [GenBank accession number [CCA57350](#) {39}; PDB accession number [5DWN](#)], with the active site substantially conserved) were also found.

Genes encoding efflux pumps from the major facilitator superfamily (MFS); the ATP-binding cassette (ABC) family, including a putative MacAB (macrolide resistance) system; the resistance-nodulation-division (RND) family, including putative AcrAB (acriflavin resistance), CmeABC, and NodT family pumps; the drug/metabolite transporter (DMT) family, including putative EmrE (ethidium bromide/methyl viologen resistance) proteins; the LysE family; and the multiantimicrobial extrusion (MATE) family were identified. Efflux pumps specifically annotated as bicyclomycin (peptide) resistance and fosmidomycin (phosphonate) resistance proteins are present.

P. mesoacidophila was also resistant to metal ions (Table 4), with the order of resistance for the transition metals tested being as follows: Mn²⁺ > Ni²⁺ > Zn²⁺ > Fe³⁺ ≈ Cu²⁺ > Co²⁺ >> Hg²⁺. *E. coli* JM109, in comparison, was sensitive to all transition metals tested, with MICs for MnCl₂ and HgCl₂ of <4 μM. Several predicted genes encoding heavy metal resistance proteins were found; these included arsenate reductase and other arsenic resistance proteins, the chromate transporter ChrAB, the cobalt/zinc/cadmium transporter CzcABCD (with a two-component regulator similar to that mediating metal resistance in *B. pseudomallei* [40]), the copper resistance proteins CopC and CopD, the HoxN/HupN/NixA family nickel/cobalt transporter, a lead/cadmium/zinc/mercury transporter, the manganese transport protein MntH, the magnesium/cobalt efflux pump CorAC, and the tellurium resistance proteins TerA, TerB, TerC, and TerD.

DISCUSSION

The 7.8-MB genome of strain ATCC 31433 was sequenced and found to comprise three chromosomal replicons and one plasmid. MLST analysis revealed that strain ATCC 31433, rather than being a pseudomonad, is a member of the *Burkholderia cepacia* complex and is closely related to *B. ubonensis*, although it may still be a distinct species. Further analysis of the bioactivity, taxonomy, and genomics of strain ATCC 31433 is warranted under its reclassification as a member of the *B. cepacia* complex.

Strain ATCC 31433 harbors many genes associated with environmental resilience and antibiotic resistance, including a wide range of efflux pumps. Antibiotic and metal susceptibility testing confirmed the resistant phenotype. Only 2 of the 24 antibiotics tested (piperacillin and ceftazidime) showed clear activity against strain ATCC 31433, and similar resistance to transition metal ions was observed. While many of the predicted heavy metal resistance genes encode efflux pumps, other detoxification systems were also seen. Several environmental *Burkholderia* strains have been described as metal resistant, although most of those strains are actually less tolerant of

metal ions than *P. mesoacidophila* (40–43). The antibiotic resistance found in strain ATCC 31433 is similar to that seen in *Burkholderia* species. Indeed, several *Burkholderia* species with high-level β -lactam resistance have been identified (9, 44). Pseudomonads are generally susceptible to monobactams, carbapenems, cefepime, aminoglycosides, and polymyxin B, whereas strain ATCC 31433 is resistant to all of these compounds, including the antipseudomonal aminoglycoside tobramycin. Resistance to monobactams may also be explained by the production of isosulfazecin by strain ATCC 31433 (1). The biosynthetic gene cluster for this metabolite, like that for sulfazecin in *P. acidophila* (19), contains efflux pumps and a β -lactamase as self-resistance mechanisms.

Despite the potential pathogenicity suggested by the genome sequence, to our knowledge there are no reported cases of strain ATCC 31433 causing disease; however, opportunistic pathogenicity toward vulnerable hosts remains an inherent trait of the *B. cepacia* complex (9). Conversely, the large number of biosynthetic gene clusters identified in the genome of strain ATCC 31433 suggests that it may be a rich source of specialized metabolites, compared to related *Burkholderia* species, and *B. ubonensis* has been shown to have activity against the pathogen *B. pseudomallei* (45). AntiSMASH (16) analysis of the strain ATCC 31433 genome identified known gene clusters for malleilactone, ornibactin, isosulfazecin, alkylhydroxyquinoline, and pyrrolnitrin biosynthesis, while revealing additional biosynthetic potential from uncharacterized biosynthetic gene clusters for polyketides, nonribosomal peptides, and other metabolites. Genes encoding bacteriocins, which are thought to represent the active principle in *B. ubonensis* activity against *B. pseudomallei* (45), were found. The biosynthesis of bulgecin is of particular interest. The biosynthetic route to the bulgecinine (4-hydroxy-5-hydroxymethylproline) core of bulgecin (12, 46) is not known but may involve direct elaboration of proline or the action of a transketolase on hydroxypyruvate and aspartate semialdehyde, prior to transfer to NRPS, glycosyltransferase, and sulfotransferase components for formation of bulgecin A itself. Several candidate genes have been identified for further investigation.

In summary, the genome sequence of strain ATCC 31433 is consistent with that of an environmental *Burkholderia* isolate from the *B. cepacia* complex. The full biosynthetic potential of this strain, including determination of the location of the gene cluster responsible for biosynthesis of the bulgecins and identification of the products of the uncharacterized gene clusters, as well as precise classification of the strain, merits further investigation. Bulgecin may prove to be a valuable tool for studying and overcoming antibiotic resistance.

MATERIALS AND METHODS

Bacterial strains and susceptibility testing. *P. mesoacidophila* ATCC 31433 was purchased from LGC Standards and was grown at 28°C in PS broth (1% [wt/vol] glucose, 0.5% [wt/vol] tryptone, 0.5% [wt/vol] meat extract, 0.5% [wt/vol] NaCl [pH 7.0]) except where indicated. *Escherichia coli* JM109 was purchased from Promega and was grown at 37°C in LB medium except where indicated. Antibiotics and metal salts were obtained from Sigma-Aldrich (Poole, United Kingdom) or Melford (Ipswich, United Kingdom). All antibiotic susceptibility testing followed the methods described by the BSAC (47), except that Mueller-Hinton II agar (Sigma-Aldrich) was used for disc diffusion assays and PS broth was used for broth microdilution assays. Breakpoints were taken from BSAC methods for antimicrobial susceptibility testing version 14 (30). For disc diffusion assays, antibiotic discs (a maximum of 3 per plate) were prepared using 5-mm filter paper discs impregnated with 5 μ l of antibiotic stock solution. Metal susceptibility testing was performed only using broth microdilution assays, with 10, 1, or 0.001 mg ml⁻¹ metal salt as the highest concentration on the plate. Antibiotic and metal susceptibility testing was performed at least in triplicate. Equivalent replicates for *P. mesoacidophila* and *E. coli* JM109 were performed on the same day using the same antibiotic stock solution.

Genome analysis. Genomic DNA was prepared from a 3-ml overnight culture of *P. mesoacidophila* in tryptic soy broth (Thermo Fisher Scientific). Cells were harvested by centrifugation and resuspended in 400 μ l of 4 M guanidine isothiocyanate solution (Thermo Fisher Scientific). DNA extraction was performed using the Maxwell 16 automated nucleic acid purification system with the Maxwell Tissue DNA purification kit (Promega), following the manufacturer's instructions.

The genome sequence was assembled from data obtained from two SMRT cells, with a PacBio RSII sequencer, using Canu 1.3 (48), polished using Quiver 2.1.0 (Pacific Biosciences, Menlo Park, CA), and circularized, where possible, using Circlator 1.2.1 (49). Gene annotations were made using PROKKA 1.11 (50) and RAST (51–53). Biosynthetic gene clusters in the genome were analyzed using AntiSMASH 3.0 (16). Graphical genome comparisons were obtained with EasyFig 2.2.2, and the ANI was calculated with

pyani 0.2.1. A virtual machine hosted by the Cloud Infrastructure for Microbial Bioinformatics (CLIMB) consortium (54) was used to perform genome assembly, annotation, and comparisons.

The sequences of six MLST alleles (*trpB*, *recA*, *lepA*, *gyrB*, *gltB*, and *atpD*) from *P. mesoacidophila* and six available *B. ubonensis* genomes were concatenated and aligned. Publicly available complete *B. ubonensis* genomes, deposited by Northern Arizona University, were downloaded from NCBI BioProject accession number PRJNA279182. The corresponding loci from 26 *Burkholderia* species were downloaded from the *Burkholderia* MLST database (<http://pubMLST.org>). A phylogeny was reconstructed with Geneious 7.1.9 Tree Builder, using the Tamura-Nei genetic distance model and the neighbor-joining tree-building method.

Detection of inducible β -lactamase activity. Starter cultures of *P. mesoacidophila* and *E. coli* JM109 were each subcultured (1:50 dilution) into four 100-ml portions of fresh PS broth and were grown at 28°C. Once A_{600} values of ~ 0.4 were attained, cefoxitin was added to give final concentrations of 0, 1, 10, and 100 $\mu\text{g ml}^{-1}$. Three hours after the addition of cefoxitin, a 25-ml aliquot was removed from each flask and the cells were lysed by sonication. The crude cellular lysate (100 μl) was then added to 200 μM nitrocefin solution in 50 mM sodium cacodylate buffer (pH 7.0) containing 100 μM ZnCl_2 and 150 mM NaCl (900 μl), and the initial rate of hydrolysis was recorded by monitoring the decrease in absorbance at 482 nm. Three repeats were obtained for each initial rate of hydrolysis, and the entire experiment was performed in triplicate.

Detection of bulgecin activity. A 48-h culture of *P. mesoacidophila* in PS broth was subcultured (1:50 dilution) into PF broth (3% glycerol, 0.5% [wt/vol] tryptone, 0.5% [wt/vol] meat extract, 0.5% [wt/vol] NaCl, 0.1% [wt/vol] glucose, 0.1% [wt/vol] cysteine [pH 7.0]) and incubated for 78 h at 28°C, at 200 rpm. Cells were removed by centrifugation, and nutrients were replenished in the supernatant solution by the addition of 0.8% (wt/vol) nutrient broth 3 (i.e., 0.5% [wt/vol] tryptone and 0.3% [wt/vol] meat extract) (Sigma-Aldrich), 2% (wt/vol) glycerol, and 0.1% (wt/vol) glucose. All broths were readjusted to pH 7 and held at 4°C overnight.

An overnight culture of *E. coli* JM109 in LB medium was subcultured (1:50 dilution) into fresh LB medium and grown at 37°C to an A_{600} value of ~ 0.3 , at 250 rpm. Cultures were centrifuged and the cell pellets were resuspended in the test broths. Cultures were then grown for an additional 4 h at 37°C, at 250 rpm. Cefuroxime (final concentration, 5 $\mu\text{g ml}^{-1}$) was added to one of each pair of flasks after 30 min; 60 and 90 min after cefuroxime addition, aliquots were removed and cells were viewed by light microscopy using an Ultraphot microscope (Zeiss). These experiments were repeated four times.

Accession number(s). The assembled genome sequence has been deposited at GenBank under accession numbers CP020737 to CP020740. The corresponding raw sequence read data are available from the European Nucleotide Archive under accession number ERP022292.

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