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Living in an Extremely Polluted Environment: Clues from the Genome of Melanin-Producing *Aeromonas salmonicida* subsp. *pectinolytica* 34mel^T

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***Aeromonas salmonicida* subsp. *pectinolytica* 34mel^T can be considered an extremophile due to the characteristics of the heavily polluted river from which it was isolated. While four subspecies of *A. salmonicida* are known fish pathogens, 34mel^T belongs to the only subspecies isolated solely from the environment. Genome analysis revealed a high metabolic versatility, the capability to cope with diverse stress agents, and the lack of several virulence factors found in pathogenic *Aeromonas*. The most relevant phenotypic characteristics of 34mel^T are pectin degradation, a distinctive trait of *A. salmonicida* subsp. *pectinolytica*, and melanin production. Genes coding for three pectate lyases were detected in a cluster, unique to this microorganism, that contains all genes needed for pectin degradation. Melanin synthesis in 34mel^T is hypothesized to occur through the homogentisate pathway, as no tyrosinases or laccases were detected and the homogentisate 1,2-dioxygenase gene is inactivated by a transposon insertion, leading to the accumulation of the melanin precursor homogentisate. Comparative genome analysis of other melanogenic *Aeromonas* strains revealed that this gene was inactivated by transposon insertions or point mutations, indicating that melanin biosynthesis in *Aeromonas* occurs through the homogentisate pathway. Horizontal gene transfer could have contributed to the adaptation of 34mel^T to a highly polluted environment, as 13 genomic islands were identified in its genome, some of them containing genes coding for fitness-related traits. Heavy metal resistance genes were also found, along with others associated with oxidative and nitrosative stresses. These characteristics, together with melanin production and the ability to use different substrates, may explain the ability of this microorganism to live in an extremely polluted environment.**

Aeromonas salmonicida subsp. *pectinolytica* 34mel^T is a melanin-producing *Aeromonas* strain that degrades polypectate, which is an unusual characteristic among *Aeromonas* species (1). It was isolated from the heavily polluted water of the Riachuelo, the last part of the Matanza River, located in Buenos Aires, Argentina. This river has received the effluents of hundreds of tanneries and other industries, as well as urban sewage and fuels, for more than a century. Among the contaminants found in this environment, which has high organic matter and low dissolved oxygen contents, are hydrocarbons, polychlorinated biphenyls, pesticides, arsenic, and heavy metals, such as chromium, lead, copper, mercury, and nickel (2–4). A molecular analysis of the microbial diversity of river water and sediments from the isolation site showed the presence of bacteria belonging to several taxa. Analysis of 16S rRNA gene sequences revealed the presence of bacteria belonging to the *Beta-*, *Gamma-*, and *Epsilonproteobacteria* in the water, as well as a higher level of diversity in the sediments, in which *Alpha-*, *Beta-*, *Gamma-*, and *Deltaproteobacteria*, *Firmicutes*, and *Bacteroidetes* were detected (5).

The genus *Aeromonas*, belonging to the *Gammaproteobacteria*, currently includes around 30 species. *A. salmonicida* comprises five different subspecies (6): *A. salmonicida* subsp. *salmonicida*, *A. salmonicida* subsp. *achromogenes*, *A. salmonicida* subsp. *masoucida*, and *A. salmonicida* subsp. *smithia*, all of which isolated from fish, and *A. salmonicida* subsp. *pectinolytica*, the only subspecies isolated from the environment. A recent phylogenetic analysis of *Aeromonas* strains based on multilocus sequence typing (MLST) showed that the first four subspecies are closely related, forming a tight cluster that excludes *A. salmonicida* subsp. *pectinolytica*, indicating that it is the most phylogenetically distant subspecies (7).

The first *Aeromonas* genome sequenced was that of *A. hydrophila* ATCC 7966^T, reflecting its ability to thrive in aquatic and host environments (8), and shortly after, the genome of *A. salmonicida* A449 provided insights into the adaptations of this fish pathogen to its host (9). Other *A. salmonicida* genomes sequenced are those of *A. salmonicida* subsp. *salmonicida* strains 01-B526 (10), 2004-05MF26, and 2009-144K3 (11), *A. salmonicida* subsp. *achromogenes* AS03 (12), *A. salmonicida* subsp. *masoucida* NBRC 13784 (BAWQ01000000), and strain CBA100 (13). Annotated genome sequences of several other *Aeromonas* species, including *A.*

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caviae (14), *A. veronii* (15), *A. media* (16), *A. aquariorum* (currently *A. dhakensis*) (17), *A. molluscorum* (18), and *A. diversa* (19), are currently available.

Bacteria living in extreme environments use a variety of different strategies to deal with the challenges to which they are exposed, which include combinations of diverse physical and chemical stress factors. The survival strategies that endow bacteria with the capability to adapt to harsh conditions involve special groups of genes, many of which can be acquired by horizontal gene transfer (20, 21). The distinctive characteristics of 34mel^T, along with its ability to live in an extremely polluted environment, prompted us to sequence the genome of this microorganism. This study constitutes an in-depth comparative genomic analysis that focuses on pectin degradation, melanin synthesis, resistance to toxic compounds, and the presence of mobile genetic elements to shed light on the capability of this strain to cope with the different challenges faced in its habitat.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *Aeromonas salmonicida* subsp. *pectinolytica* strain 34mel^T (equivalent to DSM 12609^T) was isolated in 1988 from the water of the Matanza River (Riachuelo), near the Río de la Plata estuary, in Buenos Aires, Argentina (1).

For analysis of melanin production under different conditions, cells were grown on lysogeny broth (LB) (Invitrogen) agar plates or on M9 minimal medium (6 g liter^{-1} Na_2HPO_4, 3 g liter^{-1} KH_2PO_4, 0.5 g liter^{-1} NaCl, 1 g liter^{-1} NH_4Cl, 0.2 g liter^{-1} $MgSO_4{\cdot}7H_2O$, 5 mg liter^{-1} thiamine, 2 g liter^{-1} glucose, pH 7.2) agar plates supplemented with 0.3 g liter^{-1} tyrosine and/or 0.2 mM $CuSO_4$. The capability to grow in the presence of heavy metals was analyzed by testing growth on LB plates containing a maximum of 30 ppm (0.267 mM) cadmium, as $CdCl_2$; 1,000 ppm (15.3 mM) zinc, as $ZnSO_4$; 7 ppm (0.065 mM) silver, as $AgNO_3$; 5 ppm (0.025 mM) mercury, as HgCl; 100 ppm (0.483 mM) lead, as $Pb(C_2H_3O_2)_2$; or 23 ppm (0.362 mM) copper, as $CuSO_4$. All incubations were carried out at 30°C.

Genome sequencing. Whole-genome shotgun (WGS) sequencing was performed using a Roche 454 GS FLX Titanium pyrosequencer, and reads were assembled with Newbler v. 2.6 (Roche) and annotated as described previously (22). MIRA v. 4.0 (23) was employed to close some gaps between the initially obtained Newbler contigs. The joined contigs were then confirmed by PCR assays.

Comparative genome analysis. General sequence analysis was performed using the bioinformatic tools included in the RAST (Rapid Annotation using Subsystem Technology) server (24) and the IMG (Integrated Microbial Genomes) system (25), together with BLAST (Basic Local Alignment Search Tool) (26).

The genomes of *Aeromonas* strains used for comparative analysis are shown in Table 1. Other genomes used (with corresponding GenBank accession numbers) were those of *Oceanimonas* sp. GK1 (NC_016745), *Tolumonas auensis* DSM 9187 (NC_012691), *Succinivibrio dextrinosolvens* H5 (JNKL01000000), *Aliivibrio salmonicida* LFI1238 (NC_011312 and NC_011313), *Shewanella oneidensis* MR-1 (NC_004347), *Alteromonas* sp. SN2 (NC_015554), *Marinomonas mediterranea* MMB-1 (NC_015276), *Escherichia coli* O104:H4 (NC_018658), *Dickeya dadantii* 3937 (NC_014500), and *Pseudomonas aeruginosa* UCBPP-PA14 (NC_008463). Global alignments of the whole genome of 34mel^T with those of bacterial strains belonging to the genus *Aeromonas* and the related gammaproteobacteria listed above were performed using the Needleman-Wunsch algorithm (using the BLOSUM50 scoring matrix and a maximum gap open penalty of 10), which is included in the Bioinformatics Toolbox of Matlab (30). BLASTp was used to obtain the percent identities between protein sequences. Taking into account the rapidly increasing amount of sequence data, BLAST analysis was performed against the NCBI nonredundant databases for those genes and/or proteins that were analyzed in detail.

***is*DDH.** Genome-to-genome distance calculations were performed using the GGDC program, available at http://ggdc.dsmz.de/, which provides an estimation of *in silico* DNA-DNA hybridization (*is*DDH) values (31). The program supplies results obtained with three formulas. Those reported in this work are the ones recommended by the program (formula 2).

Analysis of mobile elements and virulence factors. Prediction of genomic islands (GIs) was done using IslandViewer 2 (32) and the Colombo SIGI-HMM (33) program. Criteria used to define GIs were as follows: anomalies in G+C content and codon usage, the presence of genes associated with mobile genetic elements, such as integrases, bacteriophages, transposons, and plasmids, and a loss of synteny with genomes of related strains. To determine the boundaries of genomic islands, a comparison with the genomes of all *Aeromonas* strains included in Table 1 was performed using PanSeq (34), revealing regions unique to 34mel^T, and was complemented by manual analysis. BLASTn analysis of the putative GIs was performed against the nucleotide collection and whole-genome shotgun (WGS) databases. Flanking regions were screened for tRNA genes, which are commonly associated with the integration sites of GIs.

PhiSpy (35) and PHAST (36) were used to identify prophages. The presence of plasmids was investigated with PlasmidFinder 1.2 (37), using the *Enterobacteriaceae* database and both 80% and 50% identity thresholds. The Virulence Factor Database (VFDB) was used to search for virulence factors (38).

Nucleotide sequence accession numbers. The sequences obtained through this whole-genome shotgun project have been deposited at DDBJ/EMBL/GenBank under accession number ARYZ00000000. The version described in this paper is available under accession number ARYZ00000000.2.

RESULTS AND DISCUSSION

Genomic relatedness of 34mel^T to other *Aeromonas* strains. *A. salmonicida* subsp. *pectinolytica* was first described as a new subspecies of *A. salmonicida* after a thorough phenotypic analysis complemented by DNA-DNA hybridization (DDH) experiments that included all other *A. salmonicida* subspecies and a few other *Aeromonas* species (1). The current availability of many *Aeromonas* whole-genome sequences prompted us to extend this analysis by means of *in silico* genome-to-genome comparisons (*is*DDH) that estimate DDH values. As expected, similarities of >70% were found when 34mel^T was compared to all strains of *A. salmonicida* subsp. *salmonicida*, *A. salmonicida* subsp. *masoucida*, and *A. salmonicida* subsp. *achromogenes*, further confirming that they all belong to the same species (Table 1). The *is*DDH values were almost the same as those previously obtained experimentally for intraspecies comparisons within *A. salmonicida* (1). However, *is*DDH values were lower than 70% when 34mel^T was compared to *A. salmonicida* CBA100. When the genome of this strain was analyzed against the other *A. salmonicida* strains, *is*DDH values well below 70% were obtained, suggesting that CBA100 might not belong to this species. Further comparisons suggested that CBA100 could belong to *A. bestiarum* (*is*DDH value, 78.30%).

In silico DDH values of <70% were obtained when 34mel^T was compared to representative strains of other *Aeromonas* species (Table 1). The results obtained in the present work, which are well below the species definition cutoff value, corroborate the clear interspecies difference previously observed experimentally, in which DDH values ranging from 15.9 to 34.8% were obtained when 34mel^T was compared to other *Aeromonas* species (1).

In a recent study (27) that analyzed the genomes of a large number of *Aeromonas* strains, the authors pointed out that the

TABLE 1 *Aeromonas* genomes used for comparative analysis and *in silico* DNA-DNA hybridization values for *A. salmonicida* subsp. *pectinolytica* 34mel^T with other *Aeromonas* strains

Aeromonas strain	*is*DDH value with 34mel^T (%)	Accession no.	Genome reference
A. salmonicida subsp. *pectinolytica* 34mel^T		ARYZ00000000	22
A. salmonicida subsp. *achromogenes* AS03	73.90 ± 2.91	AMQG00000000	12
A. salmonicida subsp. *masoucida* NBRC 13784	74.40 ± 2.51	BAWQ01000000	Unpublished
A. salmonicida subsp. *salmonicida* A449	74.20 ± 2.91	CP000644	9
A. salmonicida subsp. *salmonicida* 01-B526	73.90 ± 2.51	AGVO01000000	10
A. salmonicida subsp. *salmonicida* 2009-144K3	74.10 ± 2.91	JRYV01000000	11
A. salmonicida subsp. *salmonicida* 2004-05MF26	73.90 ± 2.91	JRYW00000000	11
A. salmonicida subsp. *salmonicida* CIP 103209^T	74.10 ± 2.91	CDDW00000000	27
A. salmonicida subsp. *salmonicida* JF3224	74.10 ± 2.91	JXTA00000000	Unpublished
A. salmonicida CBA100	39.60 ± 2.51	JPWL00000000	13
A. aquariorum AAK1 (currently *A. dhakensis*)	30.90 ± 2.45	BAFL01000000	17
A. australiensis CECT 8023^T	27.80 ± 2.43	CDDH00000000	27
A. bestiarum CDC 9533-76T (equivalent to CECT 4227^T)	39.60 ± 2.51	CDDA00000000	27
A. bivalvium CECT 7113^T	26.00 ± 2.41	CDBT00000000	27
A. caviae Ae398	28.20 ± 2.43	CACP01000000	14
A. caviae CECT 838^T	28.00 ± 2.43	CDBK00000000	27
A. diversa 2478-85^T	22.00 ± 2.35	APVG00000000	19
A. encheleia CECT 4342^T	29.70 ± 2.44	CDDI00000000	27
A. enteropelogenes CECT 4487^T	27.80 ± 2.43	CDCG00000000	27
A. eucrenophila NCMB 74^T (equivalent to CECT 4224^T)	28.40 ± 2.44	CDDF00000000	27
A. fluvialis LMG 24681^T	28.60 ± 2.44	CDBO00000000	27
A. hydrophila ATCC 7966^T	31.70 ± 2.46	CP000462	8
A. hydrophila ML09-119	31.70 ± 2.46	CP005966	28
A. jandaei CECT 4228^T	27.70 ± 2.43	CDBV00000000	27
A. media WS	31.30 ± 2.46	CP007567	16
A. media RM^T (equivalent to CECT 4232^T)	30.50 ± 2.45	CDBZ00000000	27
A. molluscorum 848T^T	25.10 ± 2.40	AQGQ00000000	18
A. piscicola LMG 24783^T	41.40 ± 2.52	CDBL00000000	27
A. popoffii LMG 17541^T (equivalent to CIP 105493^T)	36.90 ± 2.49	CDBI00000000	27
A. rivuli DSM 22539^T	25.20 ± 2.40	CDBJ00000000	27
A. sanarellii LMG 24682^T	28.40 ± 2.44	CDBN00000000	27
A. simiae CIP 107798^T	22.50 ± 2.36	CDBY00000000	27
A. sobria CIP7433^T (equivalent to CECT 4245^T)	28.10 ± 2.43	CDBW00000000	27
A. taiwanensis LMG 24683^T	28.00 ± 2.43	BAWK00000000	29
A. veronii B565	28.70 ± 2.44	CP002607	15

cluster corresponding to *A. salmonicida* had a low genetic diversity, with *is*DDH values of ≥98.5%. However, that study did not include strains belonging to *A. salmonicida* subsp. *masoucida* or *A. salmonicida* subsp. *pectinolytica*. A comparison of the sequenced strain belonging to *A. salmonicida* subsp. *masoucida* with strains of *A. salmonicida* subsp. *salmonicida* revealed an *is*DDH value comparable to those reported in the mentioned work. In contrast, the *is*DDH analysis performed in the present study, in accordance with the MLST results reported previously (7), indicated that *A. salmonicida* subsp. *pectinolytica* is the most genetically distant member of the species. The low genetic diversity reported among *A. salmonicida* strains was related to the fact that the bacteria analyzed are adapted for fish pathogenicity (27). The genetic divergence between 34mel^T, an environmental isolate, and the pathogenic strains may be related to their different lifestyles.

Pectin degradation. *A. salmonicida* subsp. *pectinolytica* owes its subspecific epithet to its ability to degrade polypectate, which is a unique feature among *Aeromonas* species (1). A search for genes related to pectin degradation in the genome of 34mel^T revealed the presence of several genes coding for pectinolytic enzymes not found in other *Aeromonas* strains but similar to those present in the plant-pathogenic bacterium *Dickeya dadantii* (formerly known as *Erwinia chrysanthemi*).

Pectin, one of the main components of the plant cell wall, is a polysaccharide consisting mostly of galacturonan chains combined with rhamnose moieties and short side chains of sugars, such as galactose, arabinose, and xylose. Pectinolytic enzymes are involved in the degradation of dead plant material, contributing to the natural carbon cycle, and they are used by plant-pathogenic bacteria and fungi for the invasion of host tissues (39, 40).

Genes coding for three pectate lyases that may be involved in the first two steps in the pectin degradation pathway were detected in a cluster unique to 34mel^T that contains all genes needed for pectin degradation (Fig. 1). All of these genes are absent in other *Aeromonas* species. The pectate lyase encoded by the K931_15166 gene has 56% amino acid identity with PelE of *D. dadantii*, a bacterium that secretes eight pectate lyases and uses the degradation products as carbon sources for growth (41, 42). The second gene in the cluster codes for an exopolygalacturonate lyase (K931_15171) with 41% identity to PelX of *D. dadantii* (43). This very uncommon enzyme, also known as pectate disaccharide-lyase, is found only in some species of *Dickeya*, *Pectobacterium*, *Vibrio*, *Klebsiella*, and *Yersinia*. The third lyase, oligogalacturonide lyase (K931_15226), shares 69% amino acid identity with Ogl of *D. dadantii*. Next to *ogl* is the gene that codes for the transcriptional regulator KdgR (K931_15221) (Fig. 1), which might act as a

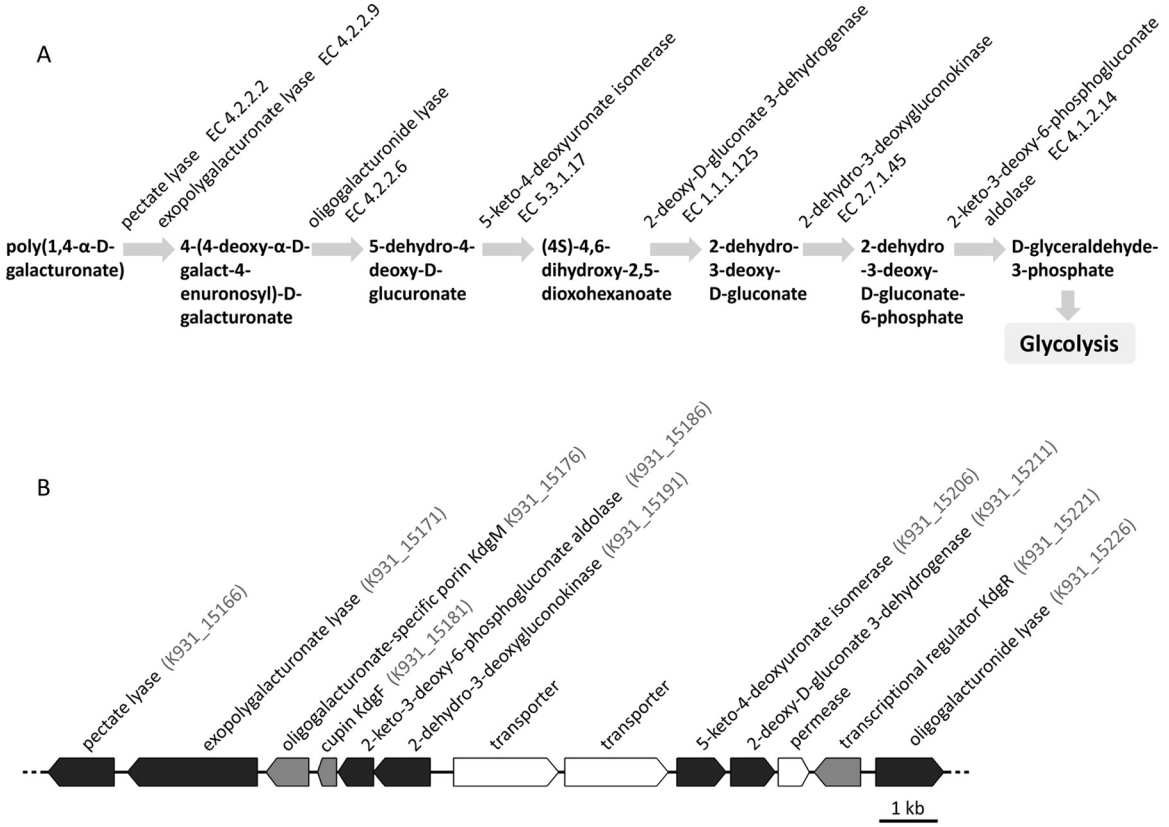

FIG 1 Pectin catabolism. (A) Pectin degradation pathway. (B) Genetic organization of the pectin degradation gene cluster. Locus tags for 34mel^T are indicated in parentheses. Genes involved in pectin degradation are shaded as follows: genes coding for pectate lyases and other enzymes of the pathway are shown in dark gray, and other genes are shown in light gray. The arrows indicate gene orientations.

general repressor of pectinolytic genes in 34mel^T. In *D. dadantii*, KdgR controls almost all steps of pectin catabolism in response to the growth phase, oxygen limitation, nitrogen starvation, and catabolite repression (43). Other genes in the cluster code for the enzymes 5-keto-4-deoxyuronate isomerase (K931_15206), 2-deoxy-D-gluconate 3-dehydrogenase (K931_15211), 2-dehydro-3-deoxygluconokinase (K931_15191), and 2-keto-3-deoxy-6-phosphogluconate aldolase (K931_15186), which catalyze degradation to the glycolytic intermediate D-glyceraldehyde-3P, indicating that 34mel^T has all the genetic information necessary for the utilization of pectin as a carbon source (Fig. 1).

The 34mel^T pectin degradation cluster also contains a gene encoding an oligogalacturonate-specific porin (K931_15176) similar to the functionally characterized porin KdgM of *D. dadantii* (44), as well as a gene encoding a cupin (K931_15181) sharing 43% identity with the pectin degradation protein KdgF of *D. dadantii*. It is worth noting that although most of the genes related to pectin degradation found in 34mel^T are similar to genes found in *D. dadantii*, a known plant pathogen, the genetic organization of the genes differs, as the pectin degradation genes in this organism are not clustered together as observed for 34mel^T.

Because *A. salmonicida* subsp. *pectinolytica* 34mel^T was isolated from the water of a highly polluted river and there are no known plant pathogens in the genus *Aeromonas*, it is possible that the capability to degrade pectin enables it to obtain nutrients from plant matter present in its environment, contributing to the nutritional versatility of this microorganism.

Melanin biosynthesis. One of the most distinctive phenotypic characteristics of 34mel^T, which is shared by all strains of *A. salmonicida* subsp. *pectinolytica*, is the production of large amounts of melanin (1), a compound known to confer protection against environmental stress (45). In rich medium, strong pigmentation was observed both in 34mel^T colonies and in the surrounding medium, with the color turning to a very dark brown due to diffusing melanin (Fig. 2). In minimal medium, melanin was not produced unless tyrosine was added, and copper ions were observed to enhance melanin production (Fig. 2). Despite the fact that melanin synthesis has been reported for several *A. salmonicida* and *A. media* strains (1, 46–48), melanin synthesis pathways have not been elucidated for these bacteria.

Melanins are complex and diverse heteropolymeric pigments formed by oxidative polymerization of phenolic and/or indolic compounds. The best-known pathway for melanin biosynthesis involves tyrosinases, which produce eumelanins (brown or black) and phaeomelanins (reddish) through the oxidation of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and dopaquinone (49). An early study that investigated melanin-like pigment production in *A. salmonicida* arrived to the conclusion that the pigment produced differed from eumelanin and phaeomelanin (46).

No genes that could code for tyrosinases were found in the genome of 34mel^T, nor were genes encoding other enzymes less frequently involved in melanin biosynthesis in bacteria, such as laccases, polyketide synthases, or 4-hydroxyphenylacetate hydroxylases. A study performed with *A. media* revealed an enzyme

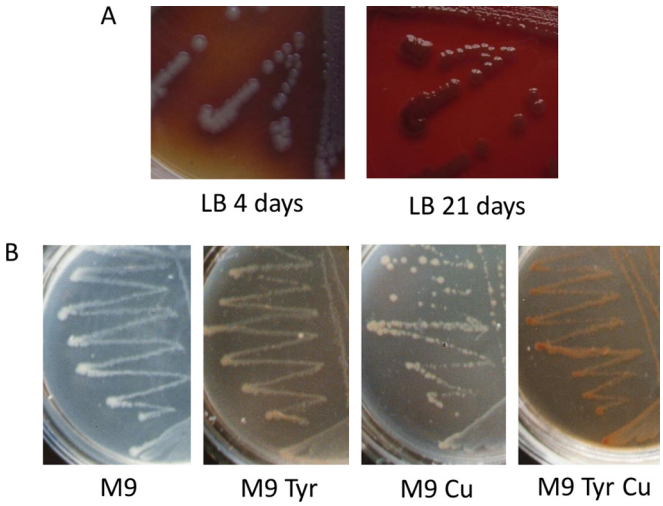

FIG 2 Melanin production by 34mel^T colonies on different media. (A) LB plates. (B) M9 plates with different additives (0.3 g liter^{-1} tyrosine and/or 0.2 mM $CuSO_4$) and incubation for 2 weeks.

(GenBank accession number ACD40043) that oxidizes L-DOPA and has little similarity to previously reported tyrosinases, and it was proposed that this enzyme is an atypical tyrosinase involved in melanin biosynthesis (50). The gene that codes for this protein is present in 34mel^T (K931_01824) and in both melanogenic and nonmelanogenic *Aeromonas* strains. When the genome of *A. media* was published, the authors indicated that it has no typical tyrosinases and mentioned that deletion of the gene previously identified as a tyrosinase gene had no effects on melanin production (16).

Pyomelanin, another kind of melanin first described for *Pseudomonas* (51), is derived from the auto-oxidation and polymerization of homogentisate, an intermediary of tyrosine catabolism, when it accumulates in the medium. A cluster of four genes is responsible for pyomelanin synthesis in *P. aeruginosa* (52) (Table 2). Three of these genes were also found in a cluster in the genome of 34mel^T: *phhA*, encoding a phenylalanine 4-monooxygenase (K931_10868); *phhB*, corresponding to a pterin-4-α-carbinolamine dehydratase (K931_10863); and *tyrR*, coding for a tran-

TABLE 2 Genes related to melanin synthesis in *A. salmonicida* 34mel^T and *Pseudomonas aeruginosa* UCBPP-PA14

Functional group and 34mel^T locus tag	Annotation for 34mel^T/putative function	Corresponding *P. aeruginosa* UCBPP-PA14 gene	
		Locus tag	Phenotype of mutant[a]
Amino acid metabolism			
K931_10868	Phenylalanine 4-monooxygenase PhhA	PA14_52990	No pigment
K931_10863	Pterin-4-α-carbinolamine dehydratase PhhB	PA14_53000	No pigment
K931_06321/K931_11063	Aromatic amino acid aminotransferase PhhC	PA14_53010	No pigment
K931_20202	4-Hydroxyphenylpyruvate dioxygenase Hpd	PA14_53070	No pigment
Nucleotide biosynthesis			
K931_13221	Dihydroorotase	PA14_05250	Reduced
K931_05586	Dihydroorotate dehydrogenase 2	PA14_24640	Reduced
K931_04105	Orotate phosphoribosyltransferase	PA14_70370	Reduced
K931_15296	Orotidine 5′-phosphate decarboxylase	PA14_26890	Reduced
K931_14303	Carbamoyl phosphate synthase	PA14_62910	Reduced
Transcription and regulation			
K931_10858	Transcriptional regulatory protein TyrR	PA14_52980	No pigment
Not found	Two-component response regulator	PA14_62540	Reduced
K931_05041	Two-component system nitrogen response regulator	PA14_67680	Reduced
K931_10283	Camphor resistance protein CrcB	PA14_56980	Reduced
K931_14746	RNA polymerase sigma-54 factor	PA14_57940	Reduced
Absent (frameshift)	Sensor/response regulator hybrid	PA14_10770	Reduced
Not found	Transcriptional regulator	PA14_29590	Reduced
Membrane proteins			
K931_15729	Potassium transport protein	PA14_52400	Reduced
K931_18684	Lipocalin (outer membrane lipoprotein)	PA14_67450	No pigment
K931_14716	ABC-type transporter ATP-binding protein	PA14_57880	Reduced
Other functions			
K931_04607	Hydroxy-3-methylbut-2-enyl diphosphate reductase	PA14_60330	Reduced
K931_14951	Methylcrotonoyl-coenzyme A carboxylase	PA14_38480	Reduced
Not found	Hypothetical protein	PA14_53260	Reduced
K931_06636	Aminodeoxychorismate lyase	PA14_25730	No pigment
Not found	Membrane protein	PA14_49050	No pigment
K931_01505	Putative dioxygenase	PA14_27390	Reduced
Not found	Hypothetical protein	PA14_22260	Reduced

[a] Effects of mutations in *P. aeruginosa* according to reference 52. "Reduced" indicates a reduction in pigment production.

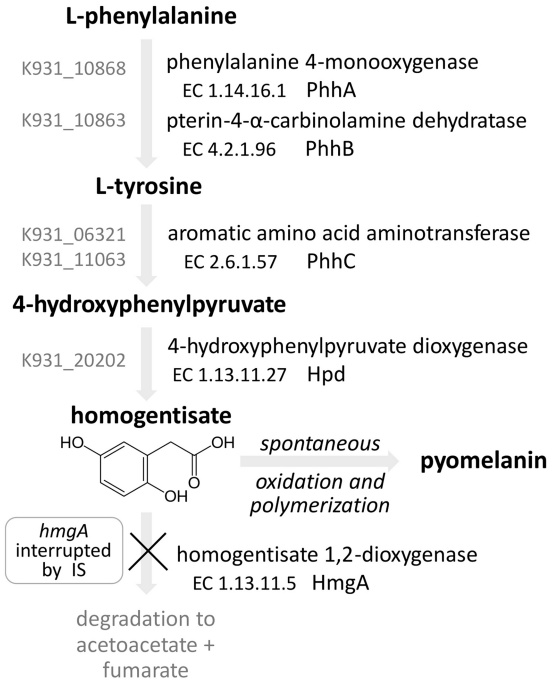

FIG 3 Melanin biosynthesis pathway. The crossed-out arrow indicates an interruption of the pathway due to the inactivation of *hmgA*, the gene that codes for the homogentisate 1,2-dioxygenase. Locus tags for the corresponding genes in 34mel^T are indicated at the left.

scriptional regulatory protein (K931_10858) (Fig. 3 and Table 2). The fourth gene in the *P. aeruginosa* cluster codes for an aromatic amino acid aminotransferase (EC 2.6.1.57). A search in the genome of 34mel^T revealed the presence of two similar genes (K931_06321 and K931_11063) located far from *phhA*, *phhB*, and *tyrR*. Another gene essential for melanin biosynthesis in *P. aeruginosa*, *hpd*, encoding a 4-hydroxyphenylpyruvate dioxygenase (52), is also present in 34mel^T (K931_20202).

During tyrosine catabolism, homogentisate is degraded to the final compounds acetoacetate and fumarate (Fig. 3). The first step is catalyzed by the homogentisate 1,2-dioxygenase, encoded by *hmgA*. Analysis of this gene in 34mel^T revealed that *hmgA* is interrupted (K931_20197 and K931_21737 correspond to the two parts of the gene) by a region containing genes that code for IstB, an ATP binding domain-containing protein (K931_21797), and a transposase (K931_21792), both of which are associated with IS*21* family insertion sequences. Previous studies showed that transposon insertions in *hmgA* resulted in accumulation of homogentisate and production of pyomelanin in *Pseudomonas* (53).

The presence of all the genes leading to the synthesis of homogentisate, together with the defective homogentisate 1,2-dioxygenase gene found in the genome of 34mel^T, suggests that this strain produces melanin by the homogentisate pathway, through the spontaneous oxidation and polymerization of the homogentisate accumulated in the medium (Fig. 3). Additionally, many other genes observed to affect melanin synthesis in *P. aeruginosa* (52) were also found in 34mel^T, distributed along the genome (Table 2).

A search for homogentisate synthesis genes in the genomes of the *Aeromonas* strains included in Table 1 revealed that all of them have the genes involved in this pathway. *A. salmonicida* A449 (9) and *A. media* WS (16) and RM^T (54) are also known to produce dark diffusible pigments. A deeper analysis of the gene coding for the homogentisate 1,2-dioxygenase in these strains revealed that *hmgA* is mutated in all of them. In *A. salmonicida* A449, *hmgA* is a pseudogene due to a frameshift caused by a single base pair deletion. This gene is interrupted by the insertion of a transposase belonging to the IS*66* family in *A. media* WS and by a nonsense codon in *A. media* RM^T.

Analysis of *hmgA* in all *Aeromonas* strains revealed that this gene contains no mutations except in 34mel^T, the three other known pigment-producing strains mentioned above, and *A. salmonicida* subsp. *salmonicida* 01-B526, 2009-144K3, 2004-05MF26, JF3224, and CIP 103209. This gene is also mutated in *A. salmonicida* subsp. *achromogenes* AS03. While no data on pigment production are available for this strain, the specific epithet of *A. salmonicida* subsp. *achromogenes* refers to a lack of pigmentation. Analysis of genes involved in the homogentisate pathway in AS03 revealed that it contains an additional mutation in *hpd*, the gene leading to homogentisate synthesis, so it is most likely nonpigmented.

Mutations in the homogentisate 1,2-dioxygenase gene have also been observed to promote melanin synthesis in *P. aeruginosa* (52). In contrast, in other bacteria that also synthesize melanin through the homogentisate pathway, such as *Vibrio cholerae* HTX-3, *Shewanella colwelliana* D, and a strain of *Hyphomonas*, the accumulation of homogentisate is due to increased amounts of the 4-hydroxyphenylpyruvate dioxygenase that leads to its synthesis, not to a mutation in the homogentisate 1,2-dioxygenase gene (55).

Taking into account that the analysis of the genomes of *Aeromonas* revealed that (i) all genes involved in the synthesis of homogentisate are present and (ii) the genes coding for the homogentisate 1,2-dioxygenase are defective in known melanogenic strains, it can be proposed that the production of melanin occurs through the oxidation and polymerization of homogentisate accumulated as a result of different mutations in the gene that codes for the enzyme that catalyzes its degradation in *A. salmonicida* 34mel^T and A449 and *A. media* WS and RM^T. The fact that four sequenced melanogenic *Aeromonas* strains have different mutations that inactivate *hmgA* indicates that these mutations arose independently and suggests that melanin production triggered by these mutations may confer a selective advantage to these bacteria.

While the present work was in the process of revision, melanin synthesis in *A. media* WS was demonstrated to occur through the homogentisate pathway (56), supporting the genetic evidence presented in this study.

Melanin biosynthesis has been observed in both pathogenic and environmental microorganisms. In pathogens, the capability to synthesize the pigment increases virulence, and in free-living bacteria, it enhances resistance against environmental stress (49). The capability of melanin to sequester metals (57) and to protect cells from UV radiation and oxidative agents (49) may help 34mel^T to survive under these and other stressful conditions encountered in the extremely polluted river water from which it was isolated.

Resistance to heavy metals. The last part of the Matanza River (Riachuelo) presents heavy metal contamination (3, 4), so 34mel^T was expected to possess the capability to deal with these pollutants. Experimental tests showed that this strain was able to grow in the presence of Ag, Cd, Hg, Pb, Zn, and Cu (data not shown).

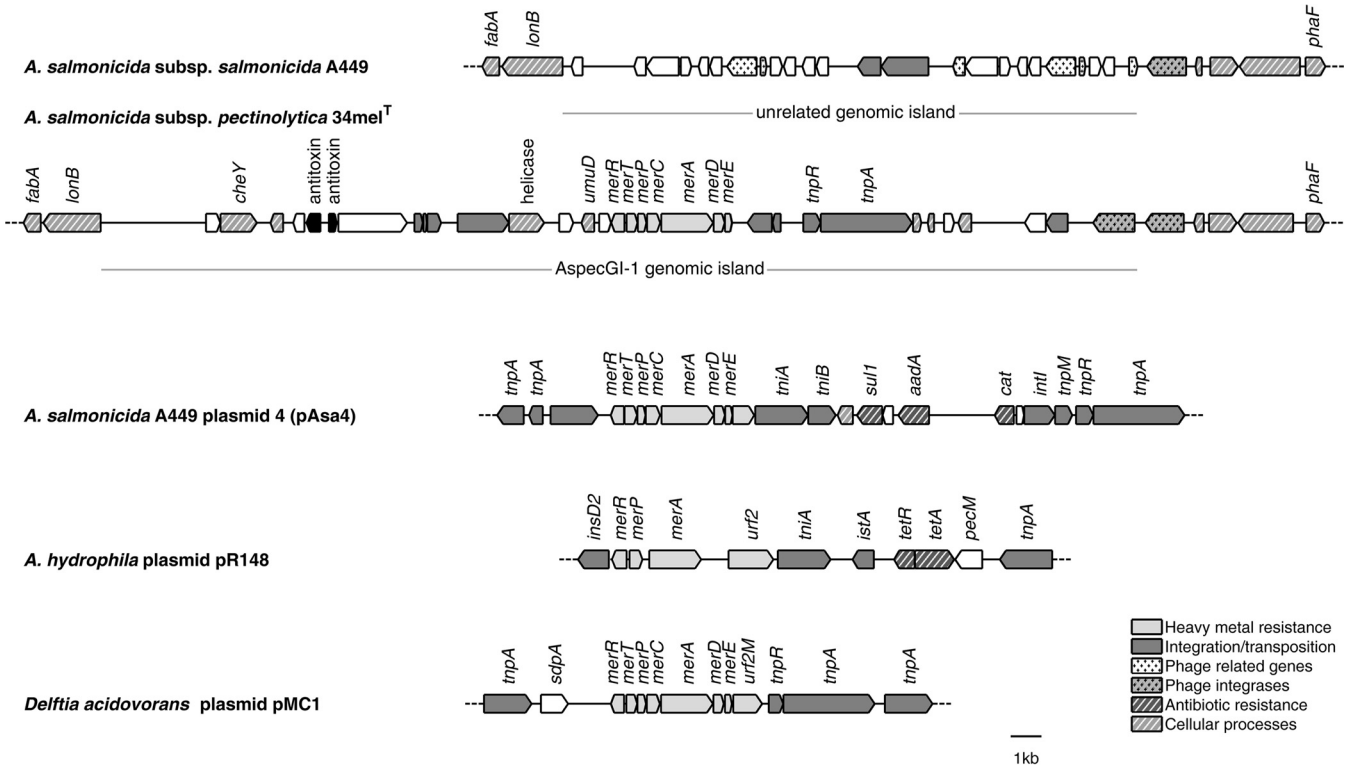

FIG 4 Organization of the *mer* region present in the genomic island AspecGI-1 of 34mel^T. Genes located at both sides of AspecGI-1 are also observed flanking a nonrelated genomic island in strain A449. The *mer* regions found in plasmids in other *Aeromonas* strains and the highly similar *mer* region of plasmid pMC1 of *D. acidovorans* are shown for comparison. Genes related to mobility and resistance to different stress factors are indicated. The arrows indicate gene orientations.

Moreover, Cu increased the synthesis of melanin (Fig. 2), and early pigmentation of the cultures was observed in the presence of 15 ppm Cd, 800 ppm Zn, or 5 ppm Ag, suggesting that the production of melanin could be used by this bacterium to cope with heavy metals.

The search for heavy metal resistance genes in the genome of 34mel^T revealed three regions containing groups of genes related to mercury, copper, silver, and arsenic resistance.

The first region contains a *mer* operon that comprises genes coding for three mercuric transport proteins (MerT, MerP, and MerC), the mercuric reductase MerA, the mercury resistance protein MerE, and the transcriptional regulators MerD and MerR (Fig. 4 and Table 3). Interestingly, sequence similarity analysis revealed that this region is almost identical (99% overall nucleotide identity) to the region containing the *mer* operon in plasmid pMC1 of the distantly related betaproteobacterium *Delftia acidovorans* (58), strongly suggesting a possible horizontal gene transfer origin. Identical *mer* regions (100% nucleotide identity) were also found in three plasmids from uncultured bacteria from agricultural soils (59). A search against other *Aeromonas* sequences revealed the presence of a *mer* operon with the same genetic organization in plasmid 4 (pAsa4) of *A. salmonicida* A449 (9), but with a lower (84%) nucleotide identity (Fig. 4). Some of the *mer* genes are also present in plasmid pR148 (89% nucleotide identity) of *A. hydrophila* (60) and plasmid pSN254b (84% nucleotide identity) of *A. salmonicida* 2004-05MF26 (11, 61), along with antimicrobial resistance genes. Highly similar (>80% nucleotide identity) *mer* operons were also observed in plasmids from both environmental strains and known pathogens, including pVS6 from *P. aeruginosa* (62), Plasmid1 from *Nitrosomonas eutropha* (63), and pECL_A from *Enterobacter cloacae* (64). Although the majority of *mer* operons similar to that of 34mel^T are located in plasmids, a high similarity (85% nucleotide identity) was also observed with the *mer* operons found in a genomic island of the multidrug resistance pathogen *Salmonella enterica* serovar Typhimurium (65) and in the chromosomes of several bacterial species, such as *Acidovorax ebreus* (66) and the enteroaggregative *E. coli* O104:H4 strain 2011C-3493 (67). However, no *mer* operons were detected in the chromosomes of *Aeromonas* strains other than 34mel^T.

The second region contains copper and silver resistance genes, including genes for periplasmic proteins, efflux pumps, and transcriptional regulators, in a region flanked by transposase genes. We detected the *cop* system, containing *copA*, encoding a multicopper oxidase; *copB*, coding for copper resistance protein B; *copC*, coding for a blue (type1) copper domain-containing protein; *copF*, coding for a heavy metal-translocating P-type ATPase; and *copRS*, encoding a two-component regulatory system (Fig. 5 and Table 3). None of these genes are present in any of the other strains of *A. salmonicida* subsp. *salmonicida*, *A. salmonicida* subsp. *achromogenes*, and *A. salmonicida* subsp. *masoucida* sequenced to date. A BLAST search for similar genes in other *Aeromonas* species revealed that *copRS* and *copF* are present in *A. hydrophila* SSU (accession no. AGWR00000000). On the other hand, the *copABC* genes were found in *A. hydrophila* SSU, *A. caviae* Ae398, and also *A. veronii* bv. sobria, in which *copA* is associated with copper tolerance, based on transposon mutant analysis (68). In addition,

TABLE 3 Genes associated with heavy metal resistance and homeostasis in 34mel^T

Functional assignment	Gene	Locus tag	Note
Mercury resistance			
Transcriptional regulator	*merR*	K931_05716	Cluster situated in AspecGI-1
Mercuric transport protein	*merT*	K931_05721	
Mercuric transport, periplasmic protein	*merP*	K931_05726	
Mercuric transport protein	*merC*	K931_05731	
Mercuric reductase	*merA*	K931_05736	
Transcriptional regulator	*merD*	K931_05741	
Mercury resistance protein	*merE*	K931_05746	
Copper and silver resistance			
Multicopper oxidase copper resistance protein	*copA*	K931_18227	Cluster unique to 34mel^T
Copper resistance protein B	*copB*	K931_18222	
Blue copper domain-containing protein	*copC*	K931_18217	
Heavy metal-translocating P-type ATPase	*copF*	K931_18197	
Two-component sensor protein	*copS*	K931_18192	
Two-component heavy metal response regulator	*copR*	K931_18187	
Copper/silver resistance periplasmic protein		K931_18172	
Copper/silver efflux system protein	*cusA*	K931_18167	
Metal binding protein	*copG*	K931_14343	
Two-component response regulator	*copR*	K931_12260	Situated in AspecGI-3
Heavy metal sensor histidine kinase	*copS*	K931_12255	
Arsenic resistance			
Arsenic operon regulator	*arsR*	K931_11408	Cluster exclusive to *A. salmonicida*
Arsenical resistance operon *trans*-acting repressor	*arsD*	K931_11403	
Arsenical pump-driving ATPase	*arsA*	K931_11398	
Arsenite efflux pump	*arsB*	K931_11393	
Arsenate reductase	*arsC*	K931_11388	
Flavin-binding monooxygenase	*arsO*	K931_11383	
Arsenical resistance regulator ArsR family transcriptional regulator	*arsR*	K931_18237	
Chromium resistance			
Chromate transport protein	*chrA*	K931_10129	
Chromate resistance regulator	*chrR*	K931_03730	
Molybdate resistance and homeostasis			
ABC-type molybdate transporter periplasmic binding protein	*modA*	K931_00175	
Molybdate ABC transporter permease	*modB*	K931_00170	
ABC-type molybdate transporter ATP-binding protein	*modC*	K931_00165	
ABC-type molybdate transporter periplasmic binding protein	*modA*	K931_07464	
ABC-type molybdate transporter permease	*modB*	K931_07469	
ABC-type molybdate transporter ATP-binding protein	*modC*	K931_07474	
Heavy metal efflux pumps and transporters			
Cobalt/zinc/cadmium efflux protein		K931_18841	Situated in AspecGI-4
Heavy metal efflux pump	*czcA*	K931_01280	
Heavy metal efflux system periplasmic protein		K931_01275	
Magnesium/cobalt efflux protein	*corB*	K931_06121	
Metal ion transporter		K931_16439	Situated in AspecGI-2
Zinc/cadmium/mercury/lead-transporting ATPase	*zntA*	K931_06296	
Cobalt/zinc/cadmium efflux system component	*czcD*	K931_06311	

genes encoding two components of copper/silver efflux pumps (K931_18167 and K931_18172) are located next to the *cop* genes (Fig. 5 and Table 3), along with *arsR*, encoding an arsenical resistance regulator (K931_18237). More genes related to copper resistance were detected in other genomic regions of 34mel^T, including a metal binding protein gene (K931_14343) similar to *copG* of *Pseudomonas putida* that is present in all *Aeromonas* strains, as well as an additional copy of *copRS* (K931_12255 and K931_12260) that is absent from the other sequenced *Aeromonas* strains. CopG has been proposed to be involved in survival in the presence of high levels of bioavailable Cu(II) (69).

The third genomic region contains a cluster of genes coding for proteins involved in arsenic detoxification and resistance that include the arsenite pump-driving ATPase ArsA, the arsenite efflux pump ArsB, the arsenate reductase ArsC, the flavin-binding monooxygenase ArsO, and the regulators ArsR and ArsD (Fig. 5 and Table 3). Comparison of these genes with those of other *Aeromonas* strains revealed that strains belonging to different *A. sal-*

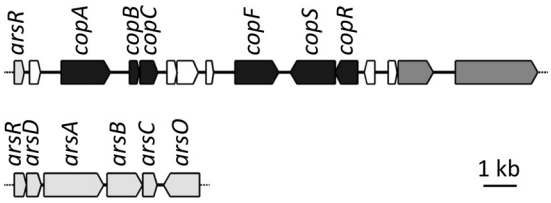

FIG 5 Organization of two genomic regions of 34mel^T containing heavy metal resistance genes. Those related to resistance to Cu are shown in black, those encoding Cu/Ag efflux pumps are shown in dark gray, and those related to resistance to As are shown in light gray. The arrows indicate gene orientations.

monicida subspecies contain the whole *ars* cluster, while most *Aeromonas* species have only some of the genes. For example, *A. hydrophila* ATCC 7966^T has four *ars* genes (8).

Apart from those located in the three regions described above, other heavy metal resistance genes were found dispersed in the genome of 34mel^T, and also in those of other *Aeromonas* strains. Among them were those encoding the chromate transporter ChrA, the chromate resistance regulator ChrR, two heavy metal efflux pumps (K931_01280 and K931_01275), the Mg/Co efflux protein CorB, the Zn/Cd/Hg/Pb-transporting ATPase ZntA, the Co/Zn/Cd efflux system component CzcD, and a metal ion transporter (K931_16439) (Table 3). Additionally, two copies of *modABC*, related to molybdate resistance and/or homeostasis, were detected. These genes code for the molybdate ABC transporter, composed of the periplasmic Mo-binding protein ModA, the permease ModB, and the ATP-binding protein ModC. The *modABC* genes were found in two different genomic regions in 34mel^T, similar to what was observed for other *Aeromonas* strains.

A previous work that studied 104 freshwater *Aeromonas* isolates belonging to 11 different species showed that most of them were sensitive to different metals, including chromium, cobalt, copper, mercury, and zinc (70), indicating that resistance to heavy metals is not widespread among these bacteria. The presence in 34mel^T of a trove of genes related to heavy metal resistance, some of which are uncommon or unique among related strains, helps us to understand the capability of this microorganism to survive in an environment with elevated heavy metal pollution.

Genomic islands. Analysis of the region surrounding the *mer* operon of 34mel^T revealed the presence of multiple genes coding for transposases and other mobile genetic elements flanking the *mer* genes that, together with codon usage bias and the lack of synteny with the genomes of related *Aeromonas* strains, indicated that this region belongs to a genomic island (Fig. 4). Genomic islands (GIs) confer important advantages relative to the lifestyle of a bacterium, contributing to genetic variability, fitness, and competitiveness in different environments (20, 21). With this in mind, a general search for GIs was performed on the genome of 34mel^T, following the criteria delineated in Materials and Methods and leading to the detection of several putative genomic islands (see Table S1 in the supplemental material).

The largest genomic island (≈34 kb), denominated AspecGI-1, contains the above-described *mer* operon (Fig. 4). AspecGI-1 could be considered a resistance and fitness island, as it also contains antitoxin genes, genes related to DNA repair and stress responses (*umuD* and *uvrD* [encodes a helicase]), and genes encoding regulators belonging to the CheY and XRE families, which may be involved in the response to environmental stimuli. Comparison with the genome of *A. salmonicida* A449 surprisingly revealed that it has an unrelated GI in the same region, containing many phage genes (Fig. 4). A high degree of synteny was observed at the sides of the GIs in both genomes, with similar (98% amino acid identity) phage integrase genes next to the GIs, suggesting the presence of a possible hot spot for GI insertions. As previously mentioned, in the other *Aeromonas* strains that contain *mer* genes, these genes are located in plasmids. In pR148 of *A. hydrophila*, plasmid 4 (pAsa4) of *A. salmonicida* A449, and plasmid pSN254b of *A. salmonicida* 2004-05MF26, the *mer* operons are situated in regions that also contain several transposase genes. However, these plasmids carry genes that provide resistance against several antibiotics (9, 60, 61), while AspecGI-1 does not contain any antibiotic resistance genes (Fig. 4; see Table S1 in the supplemental material).

Another large GI, denominated AspecGI-2, contains a large region (87% of the GI) identical (100% nucleotide identity) to the central part of Tn*1721* found in plasmid pFBAOT6 from *A. caviae* (71). However, no other genes similar to those present in pFBAOT6 and no plasmid maintenance and partitioning functions were found in the genome of 34mel^T. This GI contains genes related to stress responses along with mobile genetic elements, including a Tn*3* family transposase. Genes found in AspecGI-2 include genes coding for several transcriptional regulators, an antitoxin module, two transporter proteins, and two zinc-binding dehydrogenases and genes involved in detoxification, such as genes that code for a glutathione *S*-transferase and three methionine sulfoxide reductases that may be involved in the repair of proteins damaged by oxidative stress (72) (see Table S1). AspecGI-3 and AspecGI-4 also carry genes related to stress responses. AspecGI-3 contains the genes for a unique cold shock protein and a hydroxyisourate hydrolase (whose possible function in 34mel^T is unclear, as other enzymes involved in the catabolism of urate to allantoin were not found). AspecGI-4 contains genes coding for two cold shock-like proteins, a Co/Zn/Cd efflux protein, an outer membrane receptor for ferrienterochelin and colicins, an ABC-type transporter periplasmic binding protein, several hypothetical proteins, and two integrases (see Table S1).

Three of the GIs found in the genome of 34mel^T contained phage-related genes. An incomplete prophage, with a 41.3% G+C content, was detected in AspecGI-5 by use of the PHAST and PHISPY programs. It includes the phage attachment sites *attL* and *attR*, a tail fiber protein gene (K931_13538) similar to that of *Aeromonas* phage 65 (73), and several genes related to those of three other phages (Fig. 6). Genes encoding a portal protein (K931_13473), a prohead peptidase (K931_13478), a major capsid protein (K931_13483), a head-tail connector protein (K931_13488), and three hypothetical phage proteins (K931_13498, K931_13518, and K931_13528) similar to those of *Vibrio* phage henriette 12B8 (accession no. NC_021073.1) were also detected, together with a site-specific recombinase gene (K931_13548) related to that of *Bacillus* phage Wβ (74) and a terminase gene similar to one found in *Xanthomonas* phage phiL7 (75).

The mosaicism of this region, containing genes similar to those of several different phages, suggests that it may correspond to a novel bacteriophage. Genes encoding phage-related proteins were also found in other regions of the genome, in GIs containing mostly genes for hypothetical proteins, such as AspecGI-6 and AspecGI-7 (see Table S1 in the supplemental material). Interestingly, AspecGI-6 is located adjacent to *ssrA* (K931_00925), in the

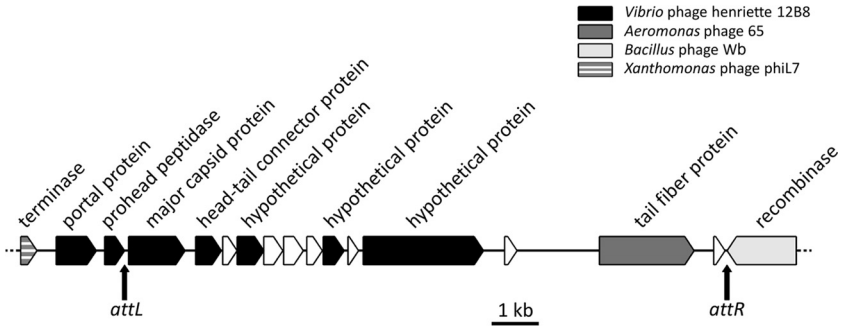

FIG 6 Genetic organization of AspecGI-5, which contains a mosaic of genes similar to those found in four previously described phages, as indicated by different shading. Phage attachment sites (*attL* and *attR*) are shown. Other genes encoding hypothetical proteins are indicated in white. The arrows indicate gene orientations.

same location observed for a phage-related GI of *A. hydrophila* PPD134/91 and GIs of other bacteria, suggesting that this locus acts as an insertion site for cryptic phages and pathogenicity islands (76).

Two metabolic GIs, AspecGI-8 and AspecGI-9, contain many genes related to cell wall synthesis. AspecGI-8 includes a novel cluster of genes involved in the biosynthesis of the O-antigen polysaccharide that differs in genetic organization compared to the corresponding genes in other *Aeromonas* strains (77). AspecGI-9 has a *Vibrio* core oligosaccharide biosynthesis gene cluster and also encodes transcriptional regulators, including a phage regulator, and carries two genes similar to those of restriction modification systems. Several additional GIs were detected, including AspecGI-10, AspecGI-11, and AspecGI-12, which are adjacent to tRNA genes, and AspecGI-13, which carries DNA repair genes (see Table S1 in the supplemental material).

A comparison of all genomic islands by using BLASTn against the WGS database confirmed that none were present in other *Aeromonas* strains, except for AspecGI-13, which was also found in *A. caviae* YL12 (accession no. JOVP00000000) and in *A. hydrophila* M062 (78).

Despite the fact that genome sequences for several *Aeromonas* strains are available, the occurrence of GIs in these bacteria was only very recently analyzed in isolates of the fish pathogen *A. salmonicida* subsp. *salmonicida* (79). GIs belong to the flexible genetic pool, as they normally carry genes that are not essential for growth but provide advantages under unfavorable conditions (20). An increased genetic versatility can contribute to the colonization of some habitats, such as those containing chemical compounds derived from anthropogenic activities, and lead to successful adaptation to changing growth conditions (21). The genetic flexibility of 34mel^T was reflected by the presence of many GIs encoding traits that could increase its fitness and survival, suggesting that acquisition of these genes has helped this microorganism to adapt to harsh environmental conditions.

Nitrogen metabolism and resistance to toxic nitrogen intermediates. In highly contaminated environments, such as the isolation site of 34mel^T, oxygen availability is scarce, favoring the growth of microorganisms that can adapt to diverse oxygen levels. *Aeromonas* species have been associated with dissimilatory nitrate reduction activity in anaerobic river sediments (80). *A. salmonicida* subsp. *pectinolytica* is a facultative aerobe that reduces nitrate to nitrite and can ferment several substrates (1). In bacteria, reduction of nitrate to nitrite can be performed by different types of nitrate reductases. Strain 34mel^T has a group of genes involved in periplasmic nitrate reduction (*nap*; K931_00200 to K931_00235) and two genes coding for the nitrate/nitrite two-component sensor regulator (K931_00245 and K931_00250), similar to those described for *A. hydrophila* ATCC 7966^T (8) and present in most of the *Aeromonas* strains currently sequenced. The physiological function of Nap is uncertain even for the well-studied denitrifying bacterium *P. aeruginosa* (81), and it has been proposed to participate in redox balancing by dissipating excess reducing power in the nondenitrifier *Rhodobacter sphaeroides* (82). The genes responsible for the conversion of nitrite to nitric oxide, an intermediary step in denitrification, were not found in the genome of 34mel^T, suggesting that it cannot carry out the complete denitrification pathway. In this metabolic scenario, the periplasmic Nap pathway of 34mel^T may be either dissimilatory or indirectly respiratory via the consumption of electrons derived from NADH, as proposed for *A. hydrophila* ATCC 7966^T (8).

Despite the absence of a complete denitrification pathway, it is interesting that the *norRVW* genes (K931_10458, K931_10463, and K931_10468), which code for proteins involved in the conversion of nitric oxide (NO) to nitrous oxide (N_2O), and *nosZ* (K931_21231), coding for the nitrous oxide reductase NosZ (EC 1.7.2.4), which catalyzes the conversion of nitrous oxide to N_2, i.e., the last step in denitrification, were found in 34mel^T. Genes coding for the transcriptional regulator NosR (K931_21226), the copper-binding periplasmic protein NosD (K931_21236), the NosF ATPase (K931_21241), the NosL lipoprotein (K931_21884), and the multicopper enzyme maturation ABC transporter NosY (K931_21889) were also detected. Among the genes involved in nitrogen metabolism commonly present in other *Aeromonas* strains are the *norRVW* genes, which are associated with detoxification processes (83). In contrast, *nos* genes are present only in 34mel^T, *A. media* WS, and *A. sanarellii* LMG 24682. NosZ removes nitrous oxide from the environment and is well studied in denitrifying bacteria (84). Nitrous oxide is a potent greenhouse gas emitted from soils and aquatic environments, including those exposed to anthropogenic activities, such as wastewater treatment plants. This compound can be produced both by nonbiological processes and as a result of nitrifying and denitrifying metabolism (85, 86). A recent study identified *nos* genes in some *Pseudomonas* strains that are not able to carry out the complete conversion of nitrate to N_2, and it has been proposed that in these bacteria these genes may have a role in the detoxification of nitrous oxide present in the environment (86). A molecular assessment of the microbial

diversity in the Matanza River showed a great abundance of potential denitrifiers (5), which are considered the main biological source of N_2O. In this context, the capability of 34mel^T to detoxify toxic nitrogen intermediates may be a selective advantage.

Pathogenicity-related genes. The genus *Aeromonas* contains many known pathogens that affect different animals, including humans. *A. salmonicida* subsp. *salmonicida* is the causative agent of furunculosis, considered an important freshwater fish disease in aquaculture, and strains belonging to *A. salmonicida* subsp. *achromogenes*, *A. salmonicida* subsp. *masoucida*, and *A. salmonicida* subsp. *smithia* are also fish pathogens (87). To date, *A. salmonicida* subsp. *pectinolytica* has been isolated solely from river water environments (1, 88), and no pathogenicity has been reported for this subspecies.

Among the main virulence factors in *Aeromonas* strains are genes corresponding to toxins and secretion systems. The type III secretion system (T3SS) genes are associated with virulence in *A. salmonicida* subsp. *salmonicida* (89). In A449, 35 of these genes are located in plasmid 5, and 1 is chromosomal (9). A study that analyzed the presence of genes related to the T3SS in different strains by dot blot hybridization reported their detection in 34mel^T (90), while a later, PCR-based assessment (91) reported that none were present in this strain. In view of these contrasting results, the genome of 34mel^T was screened for these genes. None of the 36 genes for the T3SS were found in the 34mel^T genome.

A search for genes encoding the type VI secretion system (T6SS), another virulence-associated secretion mechanism discovered in *A. hydrophila* that comprises nearly 20 proteins, revealed that T6SS genes were not present in the genome of 34mel^T, except for two copies of the gene encoding the hemolysin-coregulated protein (Hcp) and two copies of the gene encoding the valine-glycine repeat G protein (VgrG). These are structural and effector proteins of the T6SS, but it is unlikely that these proteins are functional in 34mel^T, as studies performed with *A. hydrophila* have demonstrated that the complete T6SS is necessary for the translocation of VgrG (92).

A complete T2SS gene complement (K931_09935 to K931_09990, K931_17071, and K931_17076) was found in the 34mel^T genome, with the same genetic organization as that observed in other sequenced *Aeromonas* strains. This system is responsible for the secretion of some virulence factors, such as hemolysins (93). Although hemolysis on sheep blood agar is a variable characteristic for *A. salmonicida* subsp. *pectinolytica* (1), 34mel^T is clearly hemolytic. The genes for two β-barrel pore-forming toxins, an aerolysin (K931_20432) and a hemolysin (K931_00580), similar (99% amino acid identity) to AerA and AerB of *A. salmonicida* A449, respectively, were found in the 34mel^T genome and could be responsible for its hemolytic activity.

The aerolysin AerA was early described as a lytic exotoxin in *A. hydrophila* and is one of the major virulence factors produced by this microorganism. It forms pores in the host cell membrane through a general mechanism resembling that of the *Bacillus anthracis* protective antigen (93, 94). Our preliminary experimental results have shown that production and secretion of the aerolysin of 34mel^T, which shares 96% amino acid identity with that of *A. hydrophila* ATCC 7966^T, depend on culture conditions (M. E. Pavan, E. E. Pavan, N. I. López, and M. J. Pettinari, unpublished data). A detailed analysis of the hemolysin AerB of 34mel^T revealed that it has three defined regions: a hemolysin N region, a leukocidin region, and a ricin region. Similar hemolysins were found in *A. salmonicida* subsp. *salmonicida* A449 and *A. salmonicida* subsp. *masoucida* NBRC 13784, with 99% amino acid identity, in several strains of *A. hydrophila* (91%), and in *A. aquariorum* AAK1 (91%).

Genes coding for several extracellular enzymes, such as an elastase (K931_13913), a collagenase (K931_07126), and several chitinases, were also detected in the genome of 34mel^T. The elastase of *Aeromonas veronii* bv. sobria plays an indirect role in virulence through the activation of the aerolysin (95), and the major secreted elastase of *A. hydrophila* is essential for pathogenicity (96). However, in *A. salmonicida* A449, the elastase gene (ASA_3440) contains a frameshift due to a single base pair deletion, and a pseudogene was also observed for *A. salmonicida* 01-B526, suggesting that this gene is not essential for pathogenicity in these strains. The collagenase was observed to be involved in pathogenicity in *A. veronii* RY001, as a mutant deficient in the production of this protein had reduced adhesion and invasion abilities on carp cells (97).

Three putative chitinase genes were also found in the 34mel^T genome. Two of them (K931_03026 and K931_12138) encode proteins similar to ChiB and CdxA of *A. salmonicida* A449, respectively (9). The gene coding for Chi2, another chitinase of A449, was found to be disrupted by two transposases in 34mel^T. The gene for the third chitinase found in 34mel^T (K931_03036) has no homologues in A449.

Although all these enzymes have been associated with virulence in some pathogenic bacteria, they participate in the degradation of different substrates and can serve nutritional purposes (98, 99). In 34mel^T, these enzymes could increase the range of carbon and energy sources that can be used by this microorganism.

Conclusions. Analysis of the genome of *A. salmonicida* subsp. *pectinolytica* 34mel^T revealed metabolic versatility, as it can potentially use a wide variety of substrates and tolerates the environmental challenges that this bacterium must face in the heavily contaminated river from which it was isolated. Genomic analysis shed light on the two main characteristics of this microorganism, i.e., pectin lysis and melanin synthesis. The genes for several pectinases not found in any other *Aeromonas* strain were organized in a cluster unique to 34mel^T, containing all genes needed for the complete degradation of pectin. When melanin biosynthesis pathways were analyzed, no genes coding for known enzymes leading to the synthesis of melanin through L-DOPA were detected. The discovery of a transposon insertion in the gene that codes for the homogentisate 1,2-dioxygenase led to the proposal that melanin synthesis in 34mel^T occurs through the homogentisate pathway. Genome-wide analyses of other melanin-synthesizing *Aeromonas* strains revealed different mutations in their homogentisate 1,2-dioxygenase genes, suggesting that this is the common pathway for melanin synthesis in these bacteria. Copper, silver, arsenate, and chromate resistance genes were identified, along with others related to oxidative and nitrosative stress. Some of these genes seem to have been acquired horizontally, enhancing the survival capability of this bacterium. Thirteen putative genomic islands, some of them carrying fitness-related genes, such as those involved in resistance to mercury, were found in the genome. These characteristics, together with melanin production and the ability to use different substrates, including pectin and chitin, may explain the ability of 34mel^T to thrive in an extremely polluted environment.

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