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Comparative Genomic and Transcriptomic Analyses Reveal Habitat Differentiation and Different Transcriptional Responses during Pectin Metabolism in *Alishewanella* Species

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***Alishewanella* species are expected to have high adaptability to diverse environments because they are isolated from different natural habitats. To investigate how the evolutionary history of *Alishewanella* species is reflected in their genomes, we performed comparative genomic and transcriptomic analyses of *A. jeotgali*, *A. aestuarii*, and *A. agri*, which were isolated from fermented seafood, tidal flat sediment, and soil, respectively. Genomic islands with variable GC contents indicated that invasion of prophage and transposition events occurred in *A. jeotgali* and *A. agri* but not in *A. aestuarii*. Habitat differentiation of *A. agri* from a marine environment to a terrestrial environment was proposed because the species-specific genes of *A. agri* were similar to those of soil bacteria, whereas those of *A. jeotgali* and *A. aestuarii* were more closely related to marine bacteria. Comparative transcriptomic analysis with pectin as a sole carbon source revealed different transcriptional responses in *Alishewanella* species, especially in oxidative stress-, methylglyoxal detoxification-, membrane maintenance-, and protease/chaperone activity-related genes. Transcriptomic and experimental data demonstrated that *A. agri* had a higher pectin degradation rate and more resistance to oxidative stress under pectin-amended conditions than the other 2 *Alishewanella* species. However, expression patterns of genes in the pectin metabolic pathway and of glyoxylate bypass genes were similar among all 3 *Alishewanella* species. Our comparative genomic and transcriptomic data revealed that *Alishewanella* species have evolved through horizontal gene transfer and habitat differentiation and that pectin degradation pathways in *Alishewanella* species are highly conserved, although stress responses of each *Alishewanella* species differed under pectin culture conditions.**

The diversity of *Alishewanella* species has begun to be explored only recently, since the establishment of this genus in 2000 (1). There are only 5 *Alishewanella* species with validly published names (1–5), 36 isolates from various environments, and 16S rRNA gene sequences of 27 uncultured *Alishewanella* strains, according to NCBI taxonomy. Their isolation sources include fermented foods, tidal flat sediments, plant leaf and root surfaces, soils, deserts in cold climates, sludge, permafrost soils, freshwater biofilms, metal tailings, guts of beetle larvae, lakes, wastewater, and heavy metal-resistant communities. These diverse isolation sources imply that *Alishewanella* species have a broad range of niches and high adaptability. Additionally, a predominance of *Alishewanella* species was reported for a culture-dependent analysis of a bacterial community from a lake (6).

Phylogenetic analysis of 16S rRNA genes from *Alishewanella* and taxonomically neighboring species showed that *Alishewanella* species have evolved from an ancestor dwelling in the marine environment (5). Isolation from different environments and geographic locations may indicate that *Alishewanella* species moved from marine habitats to diverse environments. Pioneering a new habitat may require acquisition of novel genetic and physiological characteristics. Therefore, strains isolated from different sources could be expected to possess distinctive biological characteristics reflecting the evolutionary process.

Habitat can determine the preference of the horizontally transferred genes between bacterial populations, and it has been hypothesized that transferred genes could render high adaptability for local interactions and adaptations. The best and simplest example is the acquisition of novel plasmids, chromosomal genes, or mobile genomic islands (GIs) and the acquisition of pathogen or antibiotic resistance (7). Another example from nonpathogenic species comparative genomic and metagenomic studies of *Prochlorococcus* spp., which identified that genetic variability between phylogenetically distinct bacterial groups occurred in genomic islands, laterally transferred via phages and differentially expressed (8).

The aims of this study were to identify the evolutionary history recorded in *Alishewanella* genomes and to determine the effects of evolutionary changes on phenotypes or transcriptional profiles. To address these aims, we first performed comparative genomic analysis using 3 *Alishewanella* species (*A. jeotgali*, *A. aestuarii*, and *A. agri*, isolated from fermented seafood, tidal flat sediment, and soil, respectively), characterized their genomic contents, and identified core and species-specific genes. Because *Bacillus* species with high average nucleotide similarity and highly conserved synteny have been reported to show apparently different phenotypes, we compared the transcriptional profiles of these 3 species to determine whether there were differences in core genes. Comparative transcriptomic analysis was conducted with cells grown on pectin as a sole carbon source because these 3 *Alishewanella* species are all able to utilize pectin as a carbon source. Research on pectin metabolism has been described for only a small number of bacterial species, and transcriptomic analysis of pectin metabolism has not been previously reported.

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MATERIALS AND METHODS

Genome data. The genomes of *A. jeotgali*, *A. aestuarii*, and *A. agri* were previously sequenced by our group and are publicly available under GenBank accession numbers AHTH00000000, ALAB00000000, and AKKU00000000, respectively (9–11). The locus tags of *A. jeotgali*, *A. aestuarii*, and *A. agri* genomes are AJE, AEST, and AGRI with 5-digit numbers, respectively.

Bioinformatics analysis. Genetic information was acquired from the NCBI GenBank database. Metabolic pathways were determined by using the KEGG pathway database. Multiple-genome alignment was conducted via Mauve (12) and a Web version of the Artemis Comparison Tools (ACT) (13). To determine species-specific genes, a reciprocal BLASTP search was performed by using protein-coding sequences at cutoff E values of >1e−10. To determine the expression levels of pectin metabolic genes, previously experimentally proven genes from *Escherichia coli*, *Bacillus*, and *Erwinia* spp. were used as reference sequences. Genomic islands were determined by GC variation in genomic loci using GC-Profile online software (http://tubic.tju.edu.cn/GC-Profile/). Expression of the intergenic regions was manually checked by using ACT (13).

Antibiotic resistance test. Antibiotic resistance was tested for ampicillin, kanamycin, gentamicin, tetracycline, chloramphenicol, rifampin, and norfloxacin. The 3 *Alishewanella* species were grown in nutrient broth, and approximately 10^5 CFU/ml was inoculated into fresh nutrient broth containing 1, 2, 5, 10, 20, 30, 40, or 50 μg/ml of antibiotics. Ampicillin was also tested at 100 μg/ml. Antibiotic resistance was determined after incubation of the cells at their optimal growth temperature (37°C for *A. jeotgali* and *A. aestuarii* and 30°C for *A. agri*) for 5 days. Resistance was defined as an optical density at 600 nm (OD_{600}) reading of >0.1.

Plate assay for pectin methylesterase activity. Pectin methylesterase (PME) activity was determined as previously described, with some modifications (14). Cells (10 μl) were deposited onto the surface of 1% pectin-containing minimal salt basal (MSB) medium (15). After 3 days of incubation, the diameters of colonies were measured. The plates were overlaid with 5 ml of a mixture containing 0.5% pectin, 50 mM potassium phosphate (pH 6.0), 0.1% Triton X-100, and 0.5% agarose. The overlaid plates were incubated at 30°C for 1 day, stained with 0.2% ruthenium red for 10 min, and destained with distilled water for 10 min. PME activity was determined as the diameter of the halo zone surrounding the colony.

Transcriptomic analysis via RNAseq. For reference total RNA, *A. jeotgali* was grown in a batch culture with 10 mM succinate-containing MSB medium under shaking conditions (220 rpm). Total RNA of *A. jeotgali* cells grown on succinate was isolated from mid-exponential-phase cells (OD_{600} = 0.4). The amount of protein was measured in succinate-grown cells when the OD_{600} reached 0.4. The protein concentration was 553.3 ± 31.2 μg/ml, which was close to that of mid-exponential-phase cells growing on pectin. *A. jeotgali*, *A. aestuarii*, and *A. agri* were grown on apple pectin to the mid-exponential phase (protein concentration of 600 μg/ml, determined by a Bradford assay [16]). The growth temperatures were 37°C for *A. jeotgali* and *A. aestuarii* and 30°C for *A. agri* according to previous reports describing their optimal temperatures. Total RNA was isolated by using an RNeasy minikit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Total RNA (10 μg) from each sample was used as starting material to prepare sequencing libraries with TruSeq RNA sample preparation kits (Illumina, USA) according to the manufacturer's instructions. This kit contained rRNA reduction procedures using biotinylated probes. One lane per sample was used for sequencing with the Illumina Genome Analyzer IIx system to generate nondirectional, single-ended, 36-bp reads. Quality-filtered reads were mapped to the *Alishewanella* genomes as reference sequences by using CLC Genomics Workbench 4.0 (CLC Bio). The relative transcript abundance was computed by counting the reads per kilobase of exon model per million mapped sequence reads (RPKM) (17). RPKM is defined as the total matched reads/(mapped reads in millions × gene length in kilobases).

Measurement of cellular oxidation. Intracellular superoxide anion generation was determined by using dihydrorhodamine (DHR) 123 (Sigma, USA) (18). The tested cells were cultivated with 10 mM succinate or 1% (wt/vol) apple pectin (the same conditions for the RNAseq analysis) in MSB medium at their optimal growth temperature. For measurement of oxidative stress in H_2O_2- and paraquat (*N*,*N*′-dimethyl-4,4′-bipyridinium dichloride)-treated cells, exponentially growing cells were treated with 0.5 or 1.0 mM H_2O_2 and paraquat for 10 min in MSB medium containing 10 mM succinate. Cells growing exponentially were washed twice and resuspended with phosphate-buffered saline (PBS). Cells were then treated with DHR 123 (2.5 μg/ml) and were incubated for 1 h under dark conditions. DHR 123-treated cells were washed and resuspended twice with PBS. The intracellular superoxide anion-mediated oxidation of DHR 123 was assayed via FACSverse flow cytometry (BD Biosciences, USA). The samples were analyzed by using a fluorescein isothiocyanate (FITC) argon ion laser for excitation. Fluorescence intensity was determined and analyzed by measuring 10,000 cell counts. BD FACSuite software was used for data analysis.

Statistical analysis. Protein concentrations and PME activity were measured from triplicate experiments and were analyzed statistically by using analysis of variance (ANOVA) and *t* tests in Microsoft Excel.

Nucleotide sequence accession number. RNAseq data were deposited in the Gene Expression Omnibus database under accession number GSE45511.

TABLE 1 General features of *Alishewanella* species genomes

Parameter	Value for *Alishewanella* species		
	A. jeotgali	*A. aestuarii*	*A. agri*
Isolation source	Fermented food	Tidal flat sediment	Landfill soil
Genome size (bp)	3,844,563	3,588,054	3,491,709
GC content (%)	50.66	50.98	50.60
Plasmid detected	None	None	None
No. of tRNA genes	64	70	68
No. of rRNA genes	6	4	3
5S	1	0[b]	0[b]
16S	1	2	1
23S	4	2	2
No. of protein-coding sequences	3,669	3,380	3223
No. (%) of protein-coding sequences with predicted function	1,728 (47.1)	2,305 (68.2)	1,625 (50.4)
No. (%) of protein-coding sequences with assigned COG[a]	2,227 (60.7)	2,370 (70.1)	2,349 (72.9)
DNA coding density (%)	88.9	90.6	91.6
Average gene length (bp)	931.6	957.4	1,008.3

[a] The COG category "function unknown (S)" was not included.
[b] The number of rRNA genes was counted from different contigs. Additional rRNA genes can be present in gapped sequences.

RESULTS AND DISCUSSION

General features of *Alishewanella* genomes. The genome sequences of 3 *Alishewanella* species were previously reported by our laboratory (9–11). However, intensive analyses had not yet been conducted. *A. jeotgali* had the largest genome (3.8 Mb) among the 3 *Alishewanella* species. GC contents of the 3 genomes were all similar (~51%). While rRNA genes are usually closely associated in terms of synteny, sometimes with tRNA, we could not determine the number of rRNAs as operons because rRNA genes were located at the ends of contig sequences. Therefore, only the copy numbers of 5S, 16S, and 23S rRNA genes are presented in Table 1.

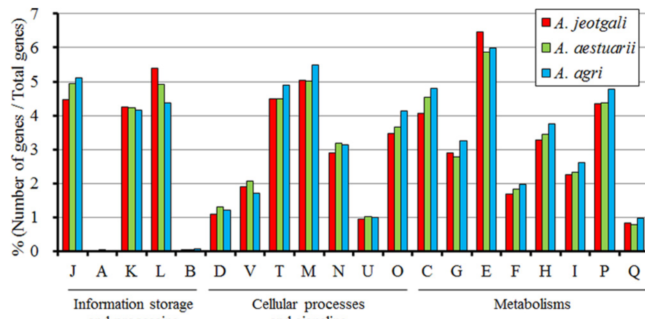

FIG 1 COG analysis of *A. jeotgali*, *A. aestuarii*, and *A. agri* genomes. COG abbreviations: J, translation, ribosomal structure, and biogenesis; A, RNA processing and modification; K, transcription; L, replication, recombination, and repair; B, chromatin structure and dynamics; D, cell cycle control, cell division, and chromosome partitioning; V, defense mechanisms; T, signal transduction mechanisms; M, cell wall/membrane/envelope biogenesis; N, cell motility; U, intracellular trafficking, secretion, and vesicular transport; O, posttranslational modification, protein turnover, and chaperones; C, energy production and conversion; G, carbohydrate transport and metabolism; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; P, inorganic ion transport and metabolism; Q, secondary metabolite biosynthesis, transport, and catabolism.

Genome data of *A. aestuarii* and *A. agri* did not contain 5S rRNA gene sequences because they were probably present in gapped genomic regions. COG analysis of the 3 genomes demonstrated that proteins related to amino acid transport and metabolism (COG abbreviation E) were the most abundant in the 3 *Alishewanella* genomes (Fig. 1). A previous report of the *A. agri* genome presumed that contig027 to contig029 could be plasmid sequences according to their sizes and paired-end sequences (11). However, more detailed analyses via reciprocal BLAST searches indicated that homologs of contig027 to contig029 were present in both the *A. jeotgali* and *A. aestuarii* genomes from AJE_03786 to AJE_04116 (73 kb) and AEST_28220 to AEST_28880, respectively. Moreover, no plasmid replication-related proteins were identified from *A. agri* contig027 to contig029. The extremely high level of coverage of genome sequences and our homology searches indicated the absence of a plasmid in *A. aestuarii*. The numbers of protein-coding sequences were 3,669, 3,380, and 3,223 in the *A. jeotgali*, *A. aestuarii*, and *A. agri* genomes, respectively. Functional predictions and COG analyses were relatively well characterized in *A. aestuarii*. The DNA coding density and average gene length were the highest in the *A. agri* genome. Average nucleotide identities were calculated to be 93.9%, 85.7%, and 85.4% for *A. jeotgali* versus *A. aestuarii*, *A. aestuarii* versus *A. agri*, and *A. agri* versus *A. jeotgali*, respectively.

Genomic islands may have imported potentially different functions or metabolic pathways. Seven and four genomic islands (GIs) were determined by GC content deviations in *A. jeotgali* and *A. agri*, while no significant variation in GC content was found in *A. aestuarii* (Table 2; see also Fig. S1 in the supplemental material). The total lengths of GIs in *A. jeotgali* and *A. agri* were 204.8 and 136.3 kb, respectively, and the greatest GC content deviations were found in GI 2 (36.3%) and GI 10 (41.9%) from *A. jeotgali* and *A. agri*, respectively, compared to the average GC content (~51%) (Tables 1 and 2). Functions of GIs were predicted based on the gene contents (Table 2). GI 1 was thought to be related to a restriction-modification system due to the presence of exonuclease (AJE_00370, AJE_00385, and AJE_00390), endonuclease (AJE_00400 and AJE_00510), a type I site-specific restriction-modification system (AJE_00425, AJE_00435, and AJE_00440 for R, M, and S subunits, respectively), and ATPase (AJE_00375 and AJE_00465). GI 3 contained many genes involved in flagellum biosynthesis and regulation. A similar gene organization was found for *Pseudoalteromonas tunicata* D2, *Idiomarina baltica* OS14, *Pseudoalteromonas haloplanktis* TAC125, *Vibrio splendidus* 12B01, the *Alteromonas macleodii* "deep ecotype," and *Shewanella* sp. strain PV-4. Interestingly, a portion of gene contents from GIs 3, 5, and 11 was involved in flagellum biosynthesis, metal transport, and sugar metabolism. Pál et al. investigated the evolution

TABLE 2 Genomic islands determined by GC content variations in the *A. jeotgali* and *A. agri* genomes[a]

GI	No. of contig(s)	Start position	Stop position	Size (bp)	No. of genes	GC content (%)	No. of hypothetical proteins	No. of transposase integrases	Predicted function(s)
Alishewanella jeotgali									
1	2	13959	76263	62,305	48	44.6	22	4	DNA restriction-modification system
2	3	996	7581	6,586	3	36.3	1	1	Not determined[b]
3	5	418566	467914	49,349	50	44.7	12	0	Flagellum biosynthesis
4	35–36	22575	12069	23,960	14	42.3	9	0	Signal transduction, DNA manipulation
5	36	12070	35419	23,350	26	54.7	2	2	Metal transporter
6	36–37	35420	16867	22,675	19	46.9	5	3	Antibiotics resistance
7	39–40	131943	17755	36,580	36	41	20	4	DNA restriction-modification system
Alishewanella agri									
8	1	142737	184463	41,727	32	43.1	7	1	Cell wall synthesis
9	1	377622	397599	19,978	29	45.1	18	0	Mixed features
10	3	19166	45148	25,983	21	41.9	6	3	Prophage, metal transport, oxidative stress defense
11	11	18059	66686	48,592	43	45	8	1	Sugar metabolism

[a] GIs were not identified in the *A. aestuarii* genome.
[b] The function of GI 3 was not predicted because there were not enough genes.

of metabolic networks of *E. coli* over the past 100 million years and concluded that most changes were due to horizontal gene transfer and that the driving force of gene evolution was changing environments (19). In this context, horizontal gene transfer may have conferred important physiological features with relevance to the environmental conditions and contributed to speciation from a common ancestor of *Alishewanella* species. The genomic loci of 20 copies of transposases in the *A. aestuarii* genome were not associated with GC content variations throughout the *A. aestuarii* genome sequences. Because horizontally transferred genes do not necessarily have different GC contents from that of the rest of genome, analyses that are more detailed may be required for *A. aestuarii* (20).

Gene contents in the low-GC-content regions implied the occurrence of horizontal gene transfer via prophage and transposases. The major features of typical phage islands were the presence of phage integrase, which performs integration of the phage elements, phage transcriptional regulators, helicase activity-possessing primase, and genes related to genome packaging, followed by many genes with unknown functions (21, 22). In accordance with previous reports, some of the low-GC-content genomic regions were associated with phage-related sequences. In *A. jeotgali*, AJE_12214, AJE_12313, and AJE_12348, located at the end of a low-GC-content region (bp 2673570 to 2710150; 36.6 kb), were phage integrase followed by a putative prophage repressor (AJE_12273) and many hypothetical proteins. In *A. agri*, AGRI_02408 and AGRI_02413, located at the end of a low-GC-content region (bp 487577 to 513560; 30.0 kb), were phage integrase family proteins followed by RNA-directed DNA polymerase (reverse transcriptase [AGRI_02393]).

Transposition of genetic materials also contributed to GC-content-variable regions in the *Alishewanella* genomes. These regions contained many copies of transposase genes and transposition-related sequences, such as the *res* subunit (AJE_10438) and cointegrate resolution protein T (AGRI_02333). All of the low-GC-content regions of the *A. agri* genome were associated with transposases. Transposition events produced pseudogenes via overlapping of a hypothetical protein AJE_01591 over tRNA-serine (bp 369529 to 370029) in *A. jeotgali* and hypothetical proteins AGRI_00850 and AGRI_01710 over tRNA-alanine (bp 184489 to 184653) and tRNA-serine (bp 378416 to 378838), respectively, in *A. agri*. A study of the distribution of pathogenicity islands (PIs) containing virulence factors such as adhesins, toxins, invasins, protein secretion systems, iron uptake systems, and others demonstrated that the GC content of PIs was often different from the average value of the chromosome and that their dispersion may be attributed to horizontal gene transfer in bacterial genomes, as evidenced by the presence of integrase determinants and other mobility loci (23). In some cases, tRNA genes (AJE_t00892 [tRNA-Met], AJE_t04154 [tRNA-Arg], AJE_t10470 [tRNA-Arg], and AJE_t12268 [tRNA-Leu]) were flanked by the ends of low-GC-content regions.

Prominent gene rearrangement in *A. jeotgali* shown in multiple-genome alignments. Multiple-genome alignments were performed with concatenated contig sequences because the order of all contigs was confirmed by paired-end sequencing. Figure S2 in the supplemental material shows the alignment results using ACT (http://www.webact.org/). The genomes of *A. aestuarii* and *A. agri* seemed to be largely conserved, lacking severe gene rearrangements. However, homologous genes of *A. aestuarii* were found throughout scattered regions of the *A. jeotgali* genome, implying more intensive gene rearrangements than those observed in *A. aestuarii* and *A. agri*. Because gene rearrangement could occur in the course of genome evolution, *A. jeotgali* may have encountered a certain environment requiring flexible adaptation (24).

Species-specific genes were horizontally transferred from a bacterial population in an overlapping habitat. Reciprocal BLASTP searches revealed 373, 386, and 238 coding sequences of low amino acid identity (E values of $> 1e-10$) to the other *Alishewanella* species *A. jeotgali*, *A. aestuarii*, and *A. agri*, respectively (see Fig. S3 in the supplemental material). We designated these proteins species-specific gene products and investigated their predicted functions to determine whether their presence provided any genetic or physiological characteristics for adapting to their different habits. Hypothetical proteins accounted for 36.2%, 85.2%, and 62.2% (135, 329, and 148 genes, respectively) of species-specific genes in *A. jeotgali*, *A. aestuarii*, and *A. agri*, respectively. The presence of multiple copies of transposase may mediate the introduction of new genes from other species or environmental DNA (9, 5, and 8 transposases in *A. jeotgali*, *A. aestuarii*, and *A. agri*, respectively). Another common feature of species-specific genes was the presence of phage-related sequences such as integrase, phage tape measure protein, coat protein, head subunit protein, and phage transcriptional regulator (20, 2, and 7 phage-related sequences in *A. jeotgali*, *A. aestuarii*, and *A. agri*, respectively). Prophage is known as a major contributor to microbial diversification (25) via genomic rearrangement, altering the fitness of the bacteria to survive (26), and transferring virulence factors (27). Species-specific genes, other than hypothetical protein, transposase, and prophage, were categorized with similar functions and are listed in Tables S1, S2, and S3 in the supplemental material.

We reasoned that species-specific genes were acquired from exogenous sources such as other bacterial populations in the vicinity. To confirm this hypothesis, we investigated the closest sequences of species-specific genes from GenBank based on a BLASTP search to identify whether there were more similar sequences from taxonomic groups other than the *Alishewanella* genus. All the species-specific genes found their closest matches in another bacterial group, as expected. E values obtained from the BLASTP results showed that species-specific genes from the 3 *Alishewanella* species had E values that were all $<10^{-10}$ and that some genes coded for identical amino acid sequences, as shown in sequence identity and coverage (see Tables S1, S2, and S3 in the supplemental material). Next, we identified the bacterial species from which the closest sequence of species-specific genes originated. *A. jeotgali*- and *A. aestuarii*-specific genes seemed to originate from *Alteromonas*, *Pseudoalteromonas*, *Rheinheimera*, *Vibrio*, *Shewanella*, and *Idiomarina* species. Their previously described isolation sources were all aquatic environments such as lake and seawater. However, many *A. agri*-specific genes seemed to originate from terrestrial environment dwellers such as *Pseudomonas*, *Geobacillus*, *Rhizobium*, and *Escherichia* spp. This result implied that habitat differentiation may have driven horizontal gene transfer from soil bacteria to *A. agri* and that accumulation of exogenously acquired genes may have resulted from the genomic evolution of *A. agri*. We also examined whether *A. jeotgali* and *A. aestuarii* harbored the same predicted function as *A. agri*-specific genes. From this analysis, we categorized *A. agri*-specific genes into 2 groups. The first group contained genes encoding proteins with annotated functions found only in *A. agri*; for example, this group con-

tained phytanoyl coenzyme A (CoA) dioxygenase (AGRI_00310) and an AbrB family transcriptional regulator (AGRI_01840), functions which *A. jeotgali* and *A. aestuarii* did not possess. The second group contained *A. agri*-specific genes having low sequence identities with those of *A. jeotgali* and *A. aestuarii* encoding proteins with the same function. For example, 3 *Alishewanella* species have multiple copies of glycosyltransferase; however, *A. agri*-specific glycosyltransferases (AGRI_00730, AGRI_00770, and AGRI_00785) are distinguished from other glycosyltransferases found in *A. jeotgali*, *A. aestuarii*, and *A. agri* by low sequence identity. BLASTP results and comparisons of the genes encoding proteins with the same predicted function suggested that *A. agri* acquired new or additional genes from other bacteria and that some portion of these horizontally transferred genes was from soil bacteria, implying that its habitat was moved from a marine environment to a soil environment.

Oxidative stress. Many oxidative stress-related genes were present in *Alishewanella* species (see Table S4 in the supplemental material). Manganese superoxide dismutase and iron superoxide dismutase, which both catalyze superoxide radicals into hydrogen peroxide and oxygen, were identified in all 3 *Alishewanella* species; however, the presence of *sodC*, the Cu-Zn superoxide dismutase, was not predicted. Catalases that decompose hydrogen peroxide to water and oxygen were present, with 3 gene copies in *A. jeotgali* and *A. agri* and 2 gene copies in *A. aestuarii*, which lacked homologs for AJE_10739 and AGRI_12426. Glutathione reductase, glutathione peroxidase, glutaredoxin, and glutaredoxin-related protein were present in *Alishewanella* genomes.

Motility. A previous study reported that *A. jeotgali* and *A. aestuarii* have motility and confirmed the presence of their single flagellum; *A. agri* did not have this phenotype (2, 3). We also confirmed their motility on agar plates (data not shown). Interestingly, RAST and PGAAP predicted 34.9-kb and 19.0-kb genomic loci, from AJE_03261 to AJE_03431 (35 genes) and from AJE_03546 to AJE_03646 (21 genes), related to flagellum biosynthesis and regulation in *A. jeotgali*, with homologs in *A. aestuarii* and *A. agri* having identical gene arrangements. Two copies of the flagellar motor protein MotA (AJE_03291 and AJE_14615) and 4 copies of MotB (AJE_00485, AJE_00495, AJE_03286, and AJE_14620) were identified. However, only AJE_03291 and AJE_03286 were most likely to be associated with other flagellar-related genes in the vicinity. An aspartic acid residue (Asp32 in *E. coli*), which was suggested to be essential for flagellar rotation via proton movement through the motor (28), was conserved in MotB proteins from the 3 *Alishewanella* species. Flagellum-specific chaperones required for exportation of structural components in a timely fashion were also located in other neighboring flagellar-related genes. The absence of motility in *A. agri*, despite the presence of all genetic components, remains to be investigated.

Antibiotic resistance in *Alishewanella* species. Experimental data performed in this study demonstrated that the 3 *Alishewanella* species could grow at ampicillin, kanamycin, gentamicin, tetracycline, chloramphenicol, rifampin, and norfloxacin concentrations of 100, 10, 2, 20, 5, 50, and 50 μg/ml, respectively. Interestingly, there were no differences in antibiotic resistance among the species. Ampicillin is a beta-lactam antibiotic that inhibits the synthesis of the cell wall. Beta-lactamase is responsible for the inactivation of beta-lactam antibiotics via cleavage of the lactam ring structure. *A. jeotgali* harbored 6 beta-lactamases, while *A. aestuarii* and *A. agri* had 5 beta-lactamases (see Table S5 in the supplemental material). AmpG and AmpE permeases, which are required for the expression of beta-lactamase in *P. aeruginosa* PAO1 and *E. coli*, were also identified in *Alishewanella* genomes (see Table S5 in the supplemental material) (29, 30). Putative TetR family transcriptional regulators of the multidrug efflux pump (AJE_11219, AEST_24320, and AGRI_12981) were located upstream of one of the beta-lactamase genes in each species (AJE_11214, AEST_24330, and AGRI_12976, respectively). The macrolide-specific efflux pump and multidrug efflux pumps could also have important roles in antibiotic resistance.

Carbon metabolism. Carbon source utilization by species was tested in MSB medium with 21 compounds (see Table S6 in the supplemental material). Interestingly, *Alishewanella* species could not utilize any aromatic compounds or hydrocarbons such as gentisate, benzoate, salicylate, naphthalene, toluene, paraffin, diesel, and hexadecane. Genes for glycolysis, the tricarboxylic acid (TCA) cycle, the pentose phosphate pathway, and the Entner-Doudoroff pathway were conserved in the 3 *Alishewanella* species (see Table S7 in the supplemental material). Naphthalene monooxygenase, the first enzyme in the naphthalene degradation pathway, was absent in the 3 *Alishewanella* species. Salicylate hydroxylase, which converts salicylate into catechol, was not identified in any of the 3 *Alishewanella* species. Gentisate 1,2-dioxygenase, which catalyzes the conversion of gentisate (2,5-dihydroxybenzoate) into maleylpyruvate, was not identified in any of the 3 *Alishewanella* species. Alkane 1-monooxygenase, which oxidizes alkane chains and produces alcohol for further degradation, was not identified. Thus, these data suggested that *Alishewanella* species seem to be unable to degrade naphthalene, salicylate, gentisate, and aliphatic hydrocarbons because of the absence of key enzymes required for their metabolism. The β-ketoadipate pathway is responsible for downstream pathways of many aromatic compounds and consists of catechol and protocatechuate branches. All genes for the catechol branch of the β-ketoadipate pathway were present in *A. jeotgali* and *A. agri* but not in *A. aestuarii* (see Table S8 in the supplemental material). The protocatechuate branch of the β-ketoadipate pathway was absent in all 3 *Alishewanella* species. The absence of key enzymes for the degradation of aliphatic and aromatic hydrocarbons implied the preference for carbohydrates rather than hydrocarbon, in terms of carbon source utilization.

Pectin metabolism. Pectin is a complex polysaccharide with a 1,4-linked α-D-galacturonic acid backbone. Pectin is a major component of the plant cell wall; hence, it is an abundant plant biomass in the natural environment (31), including coastal ocean and soil (32). Considering the preference for carbohydrates rather than hydrocarbons in carbon source utilization, the ability to utilize pectin could be quite an important feature in *Alishewanella* species. We provided apple pectin or citrus pectin as a sole carbon source and measured the growth of each species based on the increase in the total protein concentration rather than measuring the optical density because 1% pectin in MSB medium has its own turbidity. Growth curves for the 3 *Alishewanella* species showed that the concentration of protein increased faster and reached a higher level in 108 h when apple pectin was utilized (Fig. 2A). *A. agri* reached a higher protein concentration than *A. jeotgali* and *A. aestuarii*, regardless of the pectin source. The superior growth of *A. agri* on pectin was consistent with PME activity measured by ruthenium red staining (Fig. 2B). Interestingly, only *A. agri* showed PME activity on both apple and citrus pectin, whereas the PME activities of *A. jeotgali* and *A. aestuarii* were measurable only

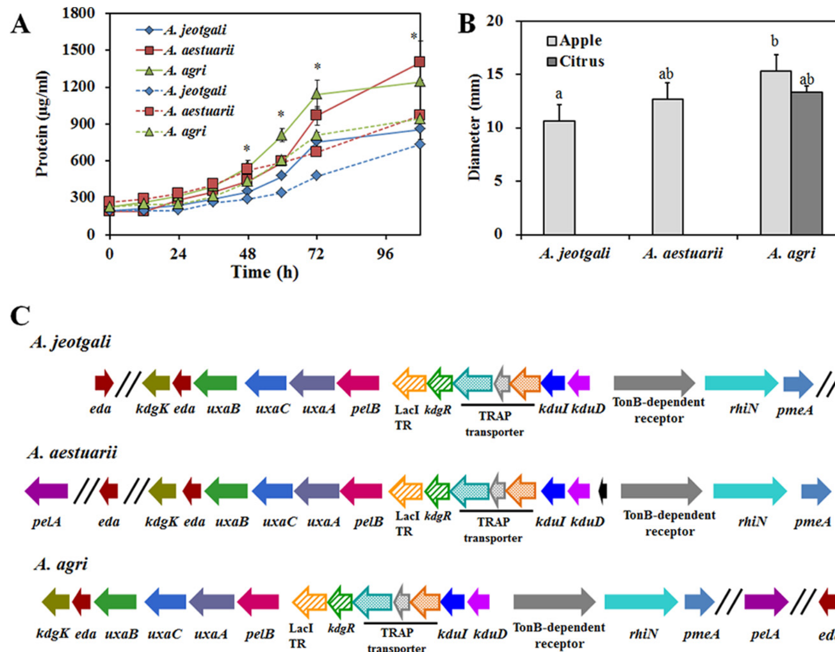

FIG 2 Characteristics of pectin utilization in *Alishewanella* species. (A) Growth curves of *Alishewanella* species on apple pectin (solid lines) and citrus pectin (dotted lines). Growth was determined by measuring the total amount of protein. (B) Pectin methylesterase (PME) activity was measured as the diameter of the halo zone after ruthenium red staining. *A. jeotgali* and *A. aestuarii* did not show PME activity on citrus pectin. (C) Gene arrangement of pectin catabolic genes. The functions of gene locus tags are summarized in Table S9 in the supplemental material. The asterisks in panel A indicate statistically significant differences, as measured by ANOVA. Bar graphs with different letters in panel B are statistically different by a *t* test ($P < 0.05$). TR, transcriptional regulator.

on citrus pectin-containing medium. The greater PME activity of *A. agri* could be one reason for its better growth on pectin, since PME deesterifies pectin to produce substrates for subsequent pectinolytic enzymes such as polygalacturonase.

Pectin metabolic genes were predicted via genome annotation and BLASTP comparison using previously described genes from *E. coli*, *Pectobacterium atrosepticum*, *B. subtilis*, and *Erwinia chrysanthemi*. The arrangement of pectin metabolism genes is shown in Table S9 in the supplemental material and in Fig. 2C. Pectin methylesterase (*pmeA*) was located at the ends of pectin metabolic loci. Deesterified pectin was degraded by pectate lyase (PelAB) and produced D-galacturonate and 4-deoxy-5-keto-L-*threo*-hexuronate at a ratio of 4:1. The transport system for 4-deoxy-5-keto-L-*threo*-hexuronate was identified experimentally in *E. chrysanthemi* and annotated *kdgT* (33). However, a homolog of *kdgT* was not found in the *Alishewanella* genomes. Downstream catabolic pathways are mediated by 4-deoxy-5-keto-uronate isomerase (*kduI*) and 2-dehydro-3-deoxy-D-gluconate-5-dehydrogenase (*kduD*), which produce 3-deoxy-D-glycero-hex-2,5-diulosonate and 2-keto-3-deoxy-D-gluconate (KDG), respectively. The transport system for D-galacturonic acid was experimentally proven and termed *exuT* (34); however, a homolog of *exuT* was not identified in the *Alishewanella* genomes. Instead, pectin metabolism loci of *Alishewanella* contained the tripartite ATP-independent transport system (TRAP) transporter family. Similar arrangements of TRAP transporter genes and pectin metabolic genes were found in *Pasteurellales*, *Vibrionales*, *Alteromonadales*, *Oceanospirillales*, and *Photorhabdus luminescens* TTO1 from the *Enterobacteriales* (35). D-Galacturonic acid is further converted to uronate isomerase (*uxaC*), D-altronate hydrolase (*uxaA*), and D-altronate oxidoreductase (*uxaB*) and produces 2-keto-3-deoxy-D-gluconate (KDG). 2-Keto-3-deoxy-D-gluconate kinase (*kdgK*) converts KDG into 2-keto-3-deoxygluconate-6-phosphate (KDPG), and 2-keto-3-deoxy-6-phosphogluconate aldolase (*kdgA*) converts KDPG into D-glyceraldehyde-3-phosphate and pyruvate. *Alishewanella* species do not have a catabolic pathway for D-glucuronate due to the absence of D-mannoate hydrolase (*uxuA*) and D-mannoate oxidoreductase (*uxuB*). Hexuronate metabolism in *E. coli* is regulated by 2 GntR family transcriptional regulators, *uxuR* and *exuR*. *uxuR* is known to control D-glucuronate metabolism, while *exuR* controls genes involved in the metabolism of D-galacturonate and D-glucuronate (35). Two transcriptional regulators were found in the *Alishewanella* genomes (LacI and IclR family), located upstream of *pelB*. The IclR family transcriptional regulator was a homolog of KdgR, the transcriptional regulator of KDG metabolism in *E. coli*. KdgR is known to regulate pectin metabolism in *Enterobacteriales* (35). Based on our comparative genomics data, we constructed the pectin metabolic pathway in *Alishewanella* species. Briefly, pectin was deesterified by pectin methylesterase and produced pectate. Pectate lyase depolymerized pectate and produced D-galacturonate and 5-keto-4-deoxy-hexuronate. These 2 products were metabolized by *uxaCAB* and *kduID*, respectively, and resulted in the formation of a common metabolite, KDG. *kdgK* and *kdgA* were responsible for the downstream pathway, and the 2 final products, D-glyceraldehyde-3-phosphate and pyruvate, were substrates for glycolysis and the TCA cycle.

Transcriptomic analysis of *Alishewanella* species utilizing pectin as a sole carbon source. Next, we performed transcriptomic analysis of the 3 *Alishewanella* species grown on apple pectin as a sole carbon source because (i) pectin metabolism was a conserved characteristic in the 3 *Alishewanella* species, despite the evidence for genetic recombination, e.g., horizontal gene transfer

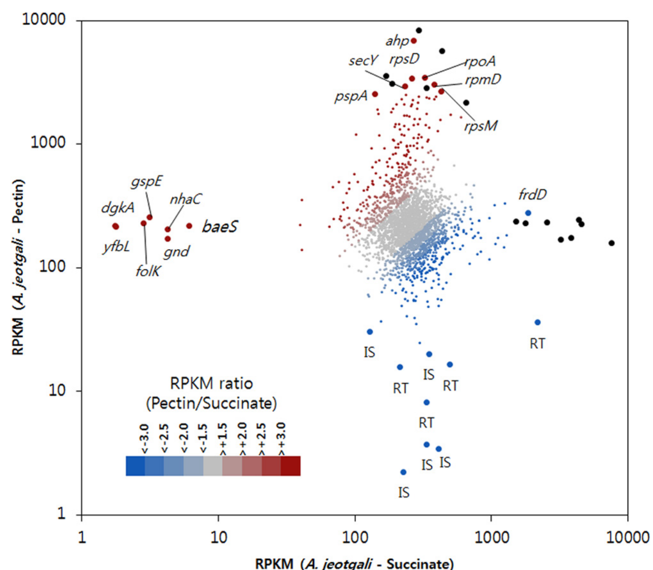

FIG 3 *xy* plot of RPKM values from *A. jeotgali* cells grown on pectin and succinate. A dot indicates a gene, and its *x* and *y* coordinates are the RPKM from succinate- and pectin-grown *A. jeotgali* cells. Fold changes (RPKM ratio of pectin/succinate) are represented with a color gradient. Gene symbols are marked next to the selected dot. Black dots indicate hypothetical proteins or proteins annotated with only general function. IS, transposase; RT, reverse transcriptase.

from other bacterial populations in their habitat, incorporation of prophage, and loss of the β-ketoadipate pathway in *A. aestuarii*; (ii) pectin could be an important carbon source for carbohydrate-preferring *Alishewanella* in the natural environment; and (iii) pectin metabolism (or D-galacturonate metabolism) has been investigated in only a limited number of bacterial species, including *E. coli*, *B. subtilis*, and *E. chrysanthemi*. Transcriptomic studies related to pectin metabolism have not yet been reported.

Transcriptomic analysis was performed via the RNAseq technique. The expression levels of genes from *A. jeotgali* grown on succinate (here called AJE_Succinate) were used as references, and RNAseq results from *A. jeotgali* grown on apple pectin (here called AJE_Pectin) were compared. RPKM values of *A. aestuarii* and *A. agri* grown on apple pectin (here called AEST_Pectin and AGRI_Pectin, respectively) were compared to those of AJE_Pectin.

The average RPKM values of AJE_Succinate, AJE_Pectin, AEST_Pectin, and AGRI_Pectin were 350.6, 357.0, 322.3, and 327.9, respectively (see Fig. S4 in the supplemental material). Median RPKM values were 250.6, 214.7, 180.7, and 236.9, respectively. Genes with RPKM values of >600 represented 1.7%, 4.6%, 5.2%, and 8.4% of the total coding sequences of AJE_Succinate, AJE_Pectin, AEST_Pectin, and AGRI_Pectin, respectively. RPKM values of the samples are represented as a color gradient ranging from 0 to 600 in Fig. 4 and 5. Figure S4 in the supplemental material shows that most genes from *A. jeotgali* and *A. aestuarii* were expressed with RPKM values of 200 to 300, while genes from *A. agri* were distributed across a wider range (RPKM values of 200 to 500).

The RPKM values of AJE_Succinate and AJE_Pectin were represented as an *xy* plot (Fig. 3). Several genes seemed to be expressed highly under only 1 condition, either pectin or succinate. Interestingly, many transposases and reverse transcriptases were highly expressed only under succinate conditions. Several functional genes were highly expressed only under pectin conditions. These genes included diacylglycerol kinase (*dgkA* [AJE_06506]), peptidase M28 (*yfbL* [AJE_07241]), 2-amino-4-hydroxy-6-hydroxymethyl-dihydropteridine pyrophosphokinase (*folK* [AJE_13045]), general secretory pathway protein E (*gspE* [AJE_09889]), 6-phosphogluconate dehydrogenase (*gnd* [AJE_16359]), Na^+/H^+ antiporter (*nhaC* [AJE_11224]), and signal transduction histidine kinase (*baeS* [AJE_12378]). To understand cellular processes in AJE_Pectin, fold changes in RPKM values of AJE_Pectin/AJE_Succinate were represented by COG categories. Figure S5 in the supplemental material shows that most genes with predicted COG categories were upregulated in AJE_Pectin. Therefore, we classified upregulated genes (>2-fold) by their functions and associated metabolic pathways or biological processes to reconstruct important cellular processes of AJE_Pectin at the transcriptional level. The following subsections provide descriptions of selected features from our transcriptomic results. Table S10 in the supplemental material summarizes the upregulated genes (>2-fold) with functional categories and RPKM values of homologs from *A. aestuarii* and *A. agri*.

Glyoxylate bypass. We investigated the expression of glycolysis, TCA cycle, pentose phosphate pathway, and Entner-Doudoroff pathway genes because succinate and downstream metabolites of pectin are substrates for these general metabolic pathways (Fig. 4). While most genes in AJE_Pectin, AEST_Pectin, and AGRI_Pectin were not expressed at levels significantly different from those in AJE_Succinate, isocitrate lyase (*aceA* [AJE_04350]) and malate synthase (*aceB* [AJE_04360]), involved in glyoxylate bypass, were the most significantly upregulated genes. Homologs of *aceA* and *aceB* from *A. aestuarii* and *A. agri* also exhibited very high RPKM values. The Leloir pathway, which converts galactose into glucose, was not responsible for the upregulation of glyoxylate bypass because *Alishewanella* genomes do not contain homologs of galactokinase and galactose-1-phosphate uridylyltransferase. Glyoxylate bypass is considered necessary for efficient utilization of carbon sources because it bypasses the CO_2-generating steps of the TCA cycle and allows cells to synthesize the building blocks of cellular components when grown on a simple carbon source such as acetate. Generally, glyoxylate bypass is upregulated when acetyl-CoA is a direct product of a metabolic pathway (36). However, glyoxylate bypass was induced by oxidative stress in several bacterial species. For example, *E. coli* experiencing superoxide stress upregulated glyoxylate bypass (37). *Pseudomonas* sp. strain TLC6-6.5-4 also upregulated its glyoxylate bypass pathway when exposed to copper stress, which caused oxidative stress (38). Proteomic and metabolomic data from *Bacillus licheniformis* showed the induction of glyoxylate bypass under conditions of peroxide stress (39). Therefore, we speculated that oxidative stress induces glyoxylate bypass in *A. jeotgali* grown on pectin, because *A. jeotgali* and *A. aestuarii* were exposed to oxidative stress when they were grown on pectin (as discussed below).

After confirming that oxidative stress was generated by hydrogen peroxide and paraquat using flow cytometry (see Fig. S6 in the supplemental material), we measured the expression levels of genes associated with the TCA cycle and glyoxylate bypass by using identical conditions to those shown in Fig. S6 in the supplemental material. Quantitative reverse transcription-PCR (qRT-PCR) results showed the relative upregulation of isocitrate lyase (*aceA*) and malate synthase (*aceB*) expression, while most other genes were downregulated (see Fig. S7 in the supplemental mate-

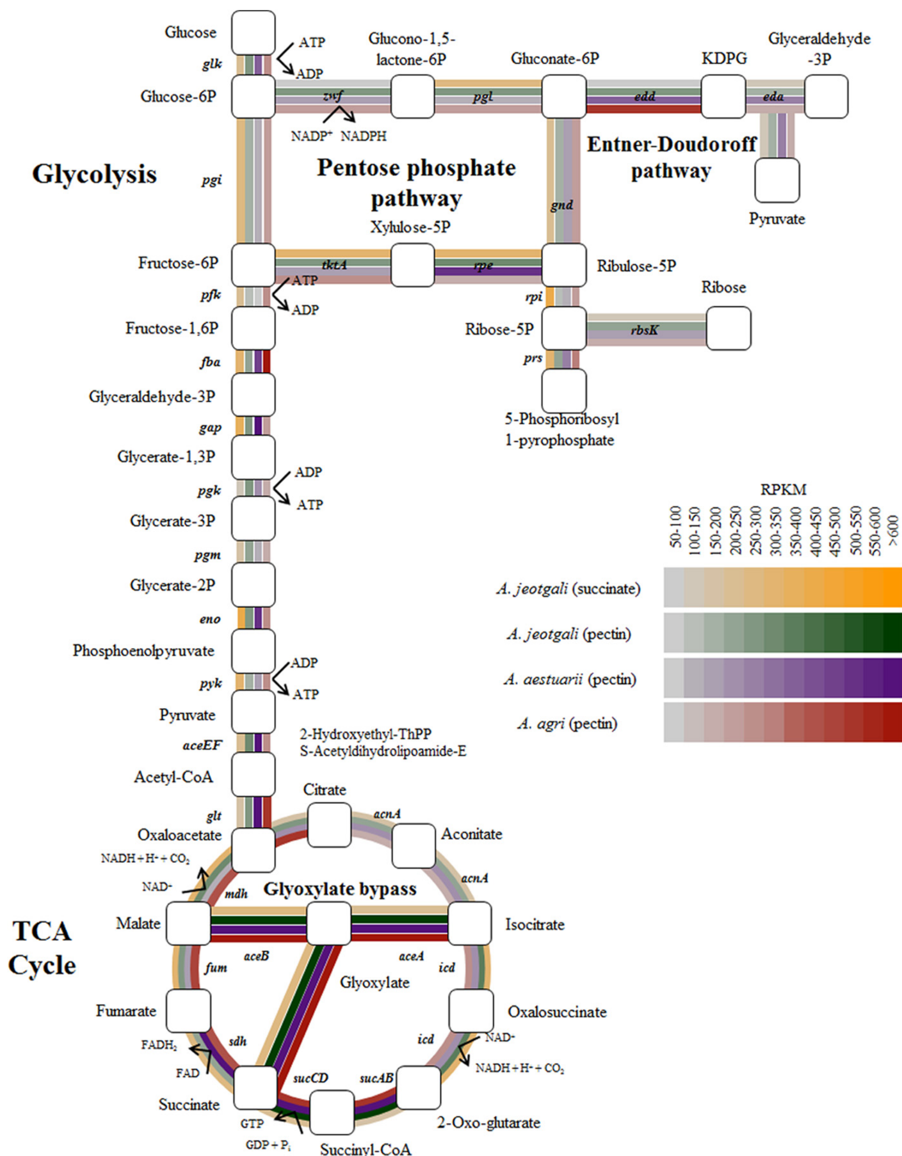

FIG 4 Expression of glycolysis, TCA cycle, pentose phosphate pathway, and Entner-Doudoroff pathway genes in succinate-grown *A. jeotgali* and pectin-grown *A. jeotgali*, *A. aestuarii*, and *A. agri* cells. Gene expression levels are represented by a color gradient based on the RPKM value. Glucose-6P, glucose-6-phosphate; FAD, flavin adenine dinucleotide; $FADH_2$, reduced flavin adenine dinucleotide.

rial). Therefore, we suggest that oxidative stress induced glyoxylate bypass in *Alishewanella* species and that oxidative stress generated during pectin utilization is responsible for highly expressed glyoxylate bypass genes. Changes in the flux of metabolites in central carbon metabolism under conditions of oxidative stress (37) or malate accumulation, producing more NADPH to cope with oxidative stress, could partially explain the upregulation of glyoxylate bypass under oxidative stress conditions (40).

Pectin metabolism. Based on the reconstructed pectin metabolic pathway, the expression of pectin metabolism-related genes was investigated. As shown in Fig. 5, pectin metabolic genes were highly expressed (RPKM values of >600) in pectin-utilizing cells, while succinate-utilizing *A. jeotgali* cells showed relatively lower expression levels of these genes. Their expression levels were higher than the top 10% of the total coding sequences. This result was expected because we supplied apple pectin as a sole carbon source. It is worthwhile to mention that the TRAP transporter system located in the pectin metabolic loci was also highly expressed. Because of the involvement of the TRAP transporter in D-galacturonic acid metabolism in several *Proteobacteria*, as shown by a comparative genomic study (35), we determined the expression level of the TRAP transporter in 3 *Alishewanella* species during the utilization of polygalacturonic acid using qRT-PCR. Figure S8 in the supplemental material shows the upregulation of the TRAP transporter in cells grown on polygalacturonic acid, suggesting their involvement in pectin metabolism. 2-Deoxy-D-gluconate 3-dehydrogenase (*kduD*) and 5-keto-4-deoxyuronate isomerase (*kduI*) did not show significantly different expression levels. Unsaturated rhamnogalacturonyl hydrolase (*rhiN* [AJE_04916]) exhibited a 3.4-fold increase in transcription. Unsaturated rhamnogalacturonyl be-

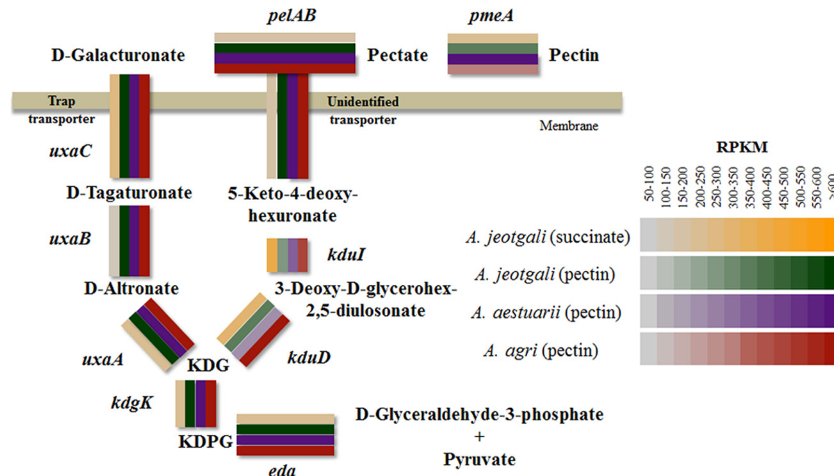

FIG 5 Expression levels of pectin metabolism pathway genes in succinate-grown *A. jeotgali* and pectin-grown *A. jeotgali*, *A. aestuarii*, and *A. agri* cells. Gene expression levels are represented by a color gradient based on the RPKM value.

longs to glycosyl hydrolase family 88, and hydrolase catalyzes the hydrolysis of unsaturated rhamnogalacturonan disaccharide to yield unsaturated D-galacturonic acid and L-rhamnose. Rhamnogalacturonan is a form of pectin and contains α-L-rhamnose in the polygalacturonate chain. In some stretches, every second monomer is L-rhamnose, and this sugar can form side chains with galactan, arabinan, or arabinogalactan. Therefore, cells may require several different kinds of pectinolytic enzymes to utilize heteropolymers of pectin.

Transcription and translation. We identified the upregulation of genes related to transcription and translation, such as RNA polymerase, sigma factor, ribosomal protein, and initiation factor. Figure 3 shows that the RPKM values of the RNA polymerase subunit (*rpoA*) and many ribosomal proteins (*rpsD*, *rpmD*, and *rpsM*) were extraordinarily high. In contrast, their RPKM values were about average when succinate was utilized as a carbon source. To exclude the possibility of early exhaustion of succinate, which would subsequently shut down translation, we compared the growth curves of *A. jeotgali* cells grown with 6, 8, or 10 mM succinate (see Fig. S9 in the supplemental material). The mean generation times were 0.94 ± 0.03 h^{-1}, 1.02 ± 0.01 h^{-1}, and 1.07 ± 0.03 h^{-1} for 6, 8, and 10 mM succinate, respectively. The maximum optical densities were 0.599 ± 0.04, 1.397 ± 0.73, and 1.411 ± 0.82 for 6, 8, and 10 mM succinate, respectively. *A. jeotgali* grew at a similar rate and reached a maximum optical density in 8 mM succinate. The growth curves showed that MSB medium containing 10 mM succinate, from which total RNA was isolated, provided enough substrate, and there were no artifacts related to a shortage of the carbon source.

Transporters. Several ion transporters, including Na^+/H^+ antiporters (*nhaB* [AJE_04480] and *nhaC* [AJE_11224]), a monovalent cation/proton antiporter (*mnhG* [AJE_06366]), an ion transport protein (AJE_09454), a Ca^{2+}/Na^+ antiporter (AJE_11724), sulfate/thiosulfate transporter subunits (*cysA* [AJE_17435] and *cysW* [AJE_17430]), and a formate/nitrite transporter (*focA* [AJE_09122]), were upregulated. The carboxyl groups and methyl esters of pectin molecules have the ability to bind oppositely charged ions, and different ion concentrations in the medium could be achieved, resulting in the differential expression of ion transport genes (41).

Oxidative stress. Many oxidative stress-related genes, including catalase (*katG* [AJE_01354]), alkyl hydroperoxide reductase (*ahpC* [AJE_08402] and *ahpF* [AJE_08407]), superoxide dismutase (*sod* [AJE_14235]), and peptide methionine sulfoxide reductase (*mrsA* [AJE_14655]) genes, were upregulated by >2-fold. The Fe(II)-trafficking protein (*yggX* [AJE_04240]) is known to protect DNA from iron-mediated oxidative damage (42) and was upregulated by 2.1-fold. The generation of oxidative stress by pectin-utilizing *Alishewanella* species was investigated by using DHR 123 (18). Nonfluorescent DHR 123 is oxidized by hydrogen peroxide, and the oxidation of DHR 123 is catalyzed by peroxidase activity. The fluorescent product, rhodamine 123, cannot cross the cell membrane and is retained in the cell. DHR 123 fluorescence was detected and visualized by flow cytometry analysis. Figure S10 in the supplemental material shows that *A. jeotgali* and *A. aestuarii* exhibited higher levels of oxidative stress when they were grown on pectin as a sole carbon source. However, interestingly, the fluorescent population of *A. agri* did not differ under the 2 conditions. The RPKM values of oxidative stress-related genes in *A. agri* were not as high as those in *A. jeotgali* and *A. aestuarii*. Therefore, *A. agri* seemed able to cope with oxidative stress via more efficient mechanisms or was more tolerant to oxidative stress than the other species. The reduced oxidative stress in *A. agri* could be the reason for its superior growth on pectin compared to *A. jeotgali* and *A. aestuarii*. Notably, we could not link oxidative stress with highly expressed glyoxylate bypass during pectin utilization in *A. agri*, unlike in the other 2 *Alishewanella* species. This can be explained by the rapid removal of reactive oxygen species before detection by DHR 123 or because the amount of oxidative stress was not high enough to be detected. Neither possibility was tested in this study.

Many upregulated proteases and chaperones (*clp*, *dnaJ*, *dnaK*, *hscA*, *groEL*, *sugE*, and *lon*) are expected to degrade damaged or misfolded proteins and assist in the proper folding of the peptide chain. Peptide methionine sulfoxide reductase (*mrsA* [AJE_14655]) was upregulated by 2.1-fold and can reduce methionine that had been oxidized by reactive oxygen species (thereby forming methionine sulfoxide) back to methionine. Relatively lower RPKM values of proteases, chaperones, and methionine sulfoxide reductase in AGRI_Pectin were confirmed.

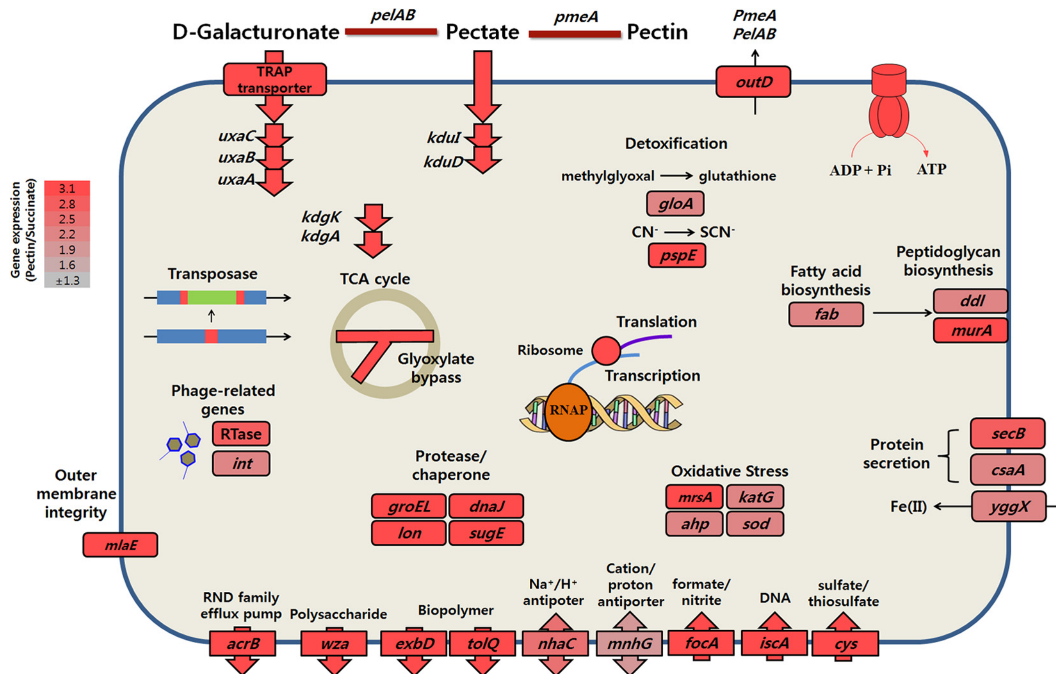

FIG 6 Upregulated cellular processes of *A. jeotgali*. Fold changes were calculated as the RPKM values of pectin-utilizing cells/succinate-utilizing cells. See the text for relevant genes and descriptions. RNAP, RNA polymerase.

Methylglyoxal detoxification. Methylglyoxal is a metabolic by-product generated naturally. It is highly toxic due to its chemical reaction with biomolecules such as proteins and nucleic acids. Lactoylglutathione lyase, also known as glyoxalase I (*gloA* [AJE_16619]), detoxifies methylglyoxal and was upregulated by 2.7-fold.

Membrane integrity. The lipids of the outer membrane are asymmetrically distributed, with lipopolysaccharides (LPSs) on the outside and phospholipids (PLs) on the inside (43). PLs are exposed to the surface of the outer membrane only in stressed cells (43). The ABC transporter membrane protein (AJE_06271) is a homolog of *mlaE* of *E. coli* and is known to maintain lipid asymmetry in the outer membrane (44). *mlaE* was upregulated by 4.7-fold in AJE_Pectin. Membrane integrity was not measured; however, the upregulation of *mlaE* indicated that the membrane integrity of *A. jeotgali* may be compromised under pectin-utilizing conditions. Upregulation of the fatty acid biosynthesis pathway (*lpxC*, *fabZ*, and *fabD*) may be required for rebuilding of the damaged cell membrane.

Motility. The upregulation of several motility genes was observed in AJE_Pectin. These genes included genes encoding chemotaxis protein, flagellar motor switch protein, flagellar M-ring protein, regulator of flagellar synthesis, and type IV pilus assembly pilin (*motA*, *spoA*, *fliF*, *flgM*, and *pilE*, respectively). Motility tests were performed with succinate- and pectin-containing motility agar plates; however, no differences in motility were observed (data not shown). A motile phenotype was not observed for *A. agri*, although motility-related genes were expressed at approximately average RPKM values for *A. agri*. We could not explain a nonmotile phenotype of *A. agri* from RNAseq data.

Gene expression of homologs between *A. aestuarii* and *A. agri*. The expression levels of homologous genes from the 3 different species (see Table S10 in the supplemental material) were not similar; that is, some genes were highly expressed in only 1 or 2 of the *Alishewanella* species, while others were highly expressed in all 3 species. This pattern prompted us to identify whether there was any correlation in gene expression levels between species. The Spearman's rank correlation value calculated from RPKM values of 2,671 core genes from *A. aestuarii* and *A. agri* was only 0.018, indicating that high RPKM values in homologous genes did not guarantee high gene expression levels in another species (see Fig. S11 in the supplemental material). This result was quite interesting, because we performed transcriptomic analysis under identical conditions and confirmed similar expression levels of genes involved in several cellular functions such as glyoxylate bypass, the pectin metabolic pathway, and transcription- and translation-related genes.

Conclusion. The *Alishewanella* genus provides a good opportunity to investigate bacterial evolutionary history, because while these species appeared to be isolated within different environments, they still contain many conserved metabolic pathways, such as the pectin metabolism pathway. There is no golden rule to determine habitat differentiation of bacterial species; however, we suggest that genomic evolution and habitat differentiation of *Alishewanella* species were relatively recent events based on our results showing relatively conserved genomic synteny, large deviations in the GC content of GIs with potential metabolic functions, and horizontally transferred soil bacterium-associated *A. agri* genes. Comparative transcriptomic analyses were conducted with pectin-metabolizing *A. jeotgali* and are depicted in Fig. 6. Comparisons of gene expression patterns in the 3 *Alishewanella* species showed different aspects of evolution (or adaptation), revealing different transcriptional profiles of homologous genes. Therefore, we speculate that the 3 *Alishewanella* species dealt with an identical situation by using different sets of genes. In addition to evolutionary context, comparative transcriptomic studies of pectin metab-

olism in the 3 different species have not been performed previously, and the relationship of pectin metabolism to diverse stress conditions has not been described in many previous studies. Therefore, this study provides valuable information for pectin metabolism in terms of global cellular processes as well as bacterial evolution.

ACKNOWLEDGMENT

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