Altered Utilization of *N*-Acetyl-D-Galactosamine by *Escherichia coli* O157:H7 from the 2006 Spinach Outbreak[▽]

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In silico analyses of previously sequenced strains of *Escherichia coli* O157:H7, EDL933 and Sakai, localized the gene cluster for the utilization of *N*-acetyl-D-galactosamine (Aga) and D-galactosamine (Gam). This gene cluster encodes the Aga phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) and other catabolic enzymes responsible for transport and catabolism of Aga. As the complete coding sequences for enzyme IIA (EIIA)^{Aga/Gam}, EIIB^{Aga}, EIIC^{Aga}, and EIID^{Aga} of the Aga PTS are present, *E. coli* O157:H7 strains normally are able to utilize Aga as a sole carbon source. The Gam PTS complex, in contrast, lacks EIIC^{Gam}, and consequently, *E. coli* O157:H7 strains cannot utilize Gam. Phenotypic analyses of 120 independent isolates of *E. coli* O157:H7 from our culture collection revealed that the overwhelming majority (118/120) displayed the expected Aga⁺ Gam[−] phenotype. Yet, when 194 individual isolates, derived from a 2006 spinach-associated *E. coli* O157:H7 outbreak, were analyzed, all (194/194) displayed an Aga[−] Gam[−] phenotype. Comparison of *aga/gam* sequences from two spinach isolates with those of EDL933 and Sakai revealed a single nucleotide change (G:C→A:T) in the *agaF* gene in the spinach-associated isolates. The base substitution in *agaF*, which encodes EIIA^{Aga/Gam} of the PTS, changes a conserved glycine residue to serine (Gly91Ser). Pyrosequencing of this region showed that all spinach-associated *E. coli* O157:H7 isolates harbored this same G:C→A:T substitution. Notably, when *agaF*⁺ was cloned into an expression vector and transformed into six spinach isolates, all (6/6) were able to grow on Aga, thus demonstrating that the Gly91Ser substitution underlies the Aga[−] phenotype in these isolates.

The genes for the utilization of *N*-acetyl-D-galactosamine (Aga) in *Escherichia coli* were initially identified by in silico analyses of the *E. coli* K-12 genome (20). The complete pathways for transport and catabolism of Aga and D-galactosamine (Gam) were established by work on *E. coli* C (Fig. 1B), which, unlike *E. coli* K-12, has the complete set of genes for the utilization of these two amino sugars (3) (Fig. 1A). The phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) discovered by Roseman and colleagues (10) is a well-studied enzyme complex responsible for the transport of a large number of carbohydrates in bacteria (17, 18), including Aga and Gam (3). The enzymes II (EII) of the PTS for Aga and Gam belong to the family of EIIs of the mannose (Man) and L-sorbose (Sor) PTS in enteric bacteria and the fructose (Fru) PTS in *Bacillus subtilis*, as they have, in addition to the IIA, IIB, and IIC domains of EII, a fourth component, the IID domain (17, 18). The integral membrane part of the EII^{Man} complex is made up of the IIC and IID proteins (3, 14, 17, 18). The IIB, IIC, and IID polypeptides for EII^{Aga} are encoded by *agaV*, *agaW*, and *agaE*, respectively, whereas the polypeptides for EII^{Gam} are encoded by *agaB*, *agaC*, and *agaD*, respectively. Notably, the IIA domain encoded by *agaF* is shared by EII^{Aga} and EII^{Gam}. The catabolic pathway of Aga and Gam is shown in Fig. 1B. Phosphorylation of these amino sugars is concomitant with uptake by their respective PTSs, thus forming Aga-

6-phosphate (Aga-6-P) or Gam-6-P. Aga-6-P is then deacetylated by the *agaA* gene product to form Gam-6-P. From this step, the catabolic pathway is common to both amino sugars. The *agaI* gene codes for a deaminase/isomerase that is responsible for converting Gam-6-P to tagatose-6-P. Further phosphorylation to tagatose-1,6-bisphosphate occurs via the action of 6-phosphofructokinase, the product of *pfkA*, a gene not part of the *aga/gam* cluster. The genes *kbaZ* and *kbaY* encode a dimeric aldolase that converts tagatose-1,6-diphosphate to dihydroxyacetone phosphate and glyceraldehyde-3-P, intermediates of the glycolytic pathway. Though the role of the *agaS* gene is not yet known, *agaR* is the repressor for this cluster of genes (3, 19). It should be pointed out that except for the aldolase activity, which has been tested in cell extracts (3), and the repressor activity, which has been demonstrated in vitro (19), the activities of the other enzymes in this pathway have been postulated based on in silico analyses (20) and similarities to the catabolic pathways for galactitol and *N*-acetyl-D-glucosamine (3). Unlike *E. coli* C, *E. coli* K-12 cannot utilize Aga or Gam (2, 3, 16). Brinkkötter and coworkers (3) showed that the Aga[−] Gam[−] phenotype of *E. coli* K-12 is due to a 2.3-kb deletion that eliminates *agaE* and *agaF* totally and truncates *agaW* and *agaA* (Fig. 1). Since *E. coli* K-12 has EIIB^{Gam}, EIIC^{Gam}, and EIID^{Gam}, the Gam⁺ phenotype can be restored by providing EIIA^{Aga/Gam} in trans from a plasmid carrying *agaF* from *E. coli* C (3).

The utilization of Aga and Gam in enterohemorrhagic *E. coli* O157:H7 strains is not well studied. We report here that *E. coli* O157:H7 strains normally display an Aga⁺ Gam[−] phenotype. In silico analyses of the *aga/gam* gene clusters of strains EDL933 and Sakai suggest that the Gam[−] phenotype is most

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FIG. 1. Comparative genetic maps of the *Aga* and *Gam* gene clusters of *E. coli* C, *E. coli* K-12, and *E. coli* O157:H7 EDL933 and Sakai (A) and the catabolic pathway for *Aga* and *Gam* in *E. coli* C (B). (A) *E. coli* C has the complete set of 13 genes: *agaR* codes for the repressor; *kbaZ* and *kbaY* code for the two subunits of tagatose-1,6-bisphosphate aldolase; *agaV*, *agaW*, and *agaE* code for EII^B, EII^C, and EII^D, respectively, of EII^{Aga}; *agaF* codes for EII^A_{Aga/Gam}; *agaA* codes for *Aga* deacetylase; *agaS* codes for a protein whose function has not been determined; *agaB*, *agaC*, and *agaD* code for EII^B, EII^C, and EII^D, respectively, of EII^{Gam}; and *agaI* codes for *Gam*-6-phosphate deaminase/isomerase. *E. coli* K-12 has a 2.3-kb deletion resulting in deletion of *agaE* and *agaF*, *agaW* truncated at the 3' end, and *agaA* truncated at the 5' end. In *E. coli* O157:H7, the annotations of *agaC* and *agaI* in strains EDL933 (shown in gray) and Sakai differ, although their sequences are the same in both strains. The eighth codon in *agaC*, which codes for glutamine in *E. coli* C, is a stop codon in *E. coli* O157:H7 because of a point mutation, C:G to T:A. In EDL933, *agaC*, shown in gray, is annotated as a 5'-truncated form, coding for a 191-amino-acid protein initiating from the in-frame 77th codon, instead of the full-length 267 amino acids as in *E. coli* C, whereas in Sakai it is not annotated. The 72nd codon of *agaI*, which codes for glutamine in *E. coli* C, is a stop codon in *E. coli* O157:H7 because of a point mutation, C:G to T:A, as in *agaC*. In EDL933 *agaI* is annotated as a split gene, shown in gray, coding for a 71-amino-acid protein from the N-terminal end and a second, 169-amino-acid protein initiating from the in-frame 83rd codon, whereas in Sakai it is not annotated. In *E. coli* C, *AgaI* is a 251-amino-acid protein. The maps are not drawn to scale. (B) In *E. coli* C, *Aga* and *Gam* are transported into the cell with their concomitant phosphorylation by the EII^{Aga} and EII^{Gam} PTSs, respectively, forming *Aga*-6-P and *Gam*-6-P. *Aga*-6-P is deacetylated by deacetylase (*AgaA*), forming *Gam*-6-P. *Gam*-6-P is then deaminated and isomerized to tagatose-6-P by *AgaI*, which is then phosphorylated by phosphofructokinase (*PfkA*) to tagatose-1,6-bisphosphate. The aldolase *KbaY/KbaZ* acts on tagatose-1,6-bisphosphate to form dihydroxyacetone phosphate and glyceraldehyde-3-P.

likely due to the lack of *agaC*, the gene encoding EII^C_{Gam}. A phenotypic metabolic microarray screening of 120 isolates from our culture collection of *E. coli* O157:H7 strains showed that all (120/120) were indeed *Gam*[−], and the vast majority of these (118/120) were *Aga*⁺. The preponderance of an *Aga*⁺ *Gam*[−] phenotype among these *E. coli* O157:H7 strains differed markedly from the phenotype of isolates obtained from a 2006 spinach-associated *E. coli* O157:H7 outbreak of human disease in that none (0/194) of the clinical or spinach-derived isolates displayed this phenotype. Rather, all of these isolates displayed an *Aga*[−] *Gam*[−] phenotype. Sequence analyses of two spinach outbreak isolates revealed only one nucleotide change (G:C→A:T) in the *agaF* gene when the *aga/gam* gene regions of these isolates were compared with those of the EDL933 and Sakai strains. This base substitution results in a conserved glycine being replaced with a serine residue (Gly91Ser). As all isolates from the spinach outbreak carried this same mutation and as the *Aga*[−] phenotype of these isolates could be complemented by a wild-type *agaF* gene, we conclude that a transition point mutation underlies this carbohydrate catabolism defect.

MATERIALS AND METHODS

Bacterial strains. A total of 194 clinical and spinach-associated isolates of *E. coli* O157:H7 from the 2006 outbreak were obtained from public health laboratories of 24 states across the United States. These isolates were coded and designated EC4001 through EC4195 to ensure patient confidentiality and compliance with Institutional Review Board guidelines. The phenotypes of these isolates were compared with those of 120 strains from our reference collection of *E. coli* O157:H7 strains. Permanent cultures of all bacterial strains and isolates used in this study are stored in 8% dimethyl sulfoxide at −70°C.

PM. Metabolic profiles of various *E. coli* O157:H7 strains were determined using the phenotypic microarray (PM) system of Biolog (Hayward, CA), which allows the simultaneous screening of approximately 1,200 phenotypes (1, 26). All materials, media, and reagents for the PM system were purchased from Biolog. The system consists of 20 96-well plates (PMs 1 to 20) designed to assess growth of a bacterial strain under various conditions. That is, each of the wells of the first 10 plates (PMs 1 to 10) contains a particular carbon (PMs 1 and 2), nitrogen (PM 3), or phosphate and sulfur (PM 4) source; micronutrients (PM 5); dipeptides (PMs 6 to 8); or different pH (PM 9) and ionic (PM 10) conditions. PMs 11 to 20 contain various antibiotics and chemical inhibitors. In wells B1 of PM 2 and E12 of PM 3, *Aga* served as a carbon and nitrogen source, respectively. *Gam* is present as a nitrogen source in well E9 of plate PM 3. PM experiments were conducted using conditions exactly as developed by Biolog (26) with the following modifications: bacteria stored at −70°C, were streaked onto Luria-Bertani (LB) agar plates instead of BUG + B agar, and ferric citrate was omitted from IF-0 medium that was added to PM 3 to PM 8. The PM plates were incubated

FIG. 2. PM plot of the utilization of Aga by *E. coli* O157:H7 EDL933 and EC4045, a spinach isolate. Data derived from the increase in intensity of the color of the reduced dye A, expressed in AU (maximum of 500 AU), are plotted against time (48 h) using the PM software from Biolog. *E. coli* O157:H7 EDL933 utilizes Aga as carbon and nitrogen sources, whereas EC4045, a spinach isolate, cannot.

at 37°C in an Omnilog incubator and readings recorded for 48 h. Bacterial respiration was assessed within each well by monitoring color formation resulting from reduction of the tetrazolium violet (dye A), and the color intensity was expressed in arbitrary units (AU) with a maximum of 500 AU. Data were analyzed with Omnilog-PM software from Biolog.

Bacterial media and growth conditions. For plating experiments, bacteria were grown overnight with shaking in 5 ml LB broth at 37°C. Bacteria were then diluted 10³-fold in 0.9% NaCl and streaked onto minimal M9 agar plates (13) supplemented with 0.001% yeast extract and containing either 0.2% glucose, 20 mM Aga, or 20 mM Gam as a carbon source. When growth in liquid medium was assessed, 20 µl of an LB overnight culture was added to 5 ml of M9 minimal medium containing 20 mM Gam and incubated with shaking at 37°C for 48 h. M9 agar without NH₄Cl was used to assess whether bacterial isolates could utilize Aga as the sole nitrogen source, scoring plates for growth after 48 h of incubation at 37°C.

Sequencing. Whole-genome sequencing of two *E. coli* O157:H7 isolates (EC4042 and EC4191) from the 2006 spinach-associated outbreak was conducted at the National Bioforensics Analysis Center (NBFAC) of the Department of Homeland Security using 454 Life Sciences Technology (11).

The pyrosequencing method has been described in detail earlier (21). Briefly, pyrosequencing utilizes the pyrophosphate released following incorporation of nucleoside monophosphate in DNA synthesis reactions to generate ATP, which subsequently is the substrate for luciferase. The light emitted by luciferase is quantified and is the signal for nucleotide incorporation. Chromosomal DNAs for pyrosequencing were prepared using AutoGenprep (Holliston, MA) according to the manufacturer's protocol. Oligonucleotide primers for amplification of a 69-bp region in the *agaF* gene were designed from the sequence of the Sakai strain. Biotinylated and nonbiotinylated primers were obtained from IDT DNA (Coralville, IA). Amplification using the 5' biotinylated primer 5'-GATGCAA AAACCGGGCTGT-3' and the 3' primer 5'-TCCAGCACCATTCTCCAGTAG C-3' was carried out in 50-µl reaction mixtures containing PCR buffer with 1.5 mM MgCl₂ (Perkin-Elmer), 2.5 mM deoxynucleoside triphosphate mixture (Pharmacia), 0.25 µM of each primer pair, 1.5 µl of *Taq* DNA polymerase (Promega), and 5 µl of DNA template. All additions were done at 4°C, and amplifications were carried out under the following conditions: denaturation at 94°C for 5 min; followed by 45 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 30 s; and a final incubation at 72°C for 10 min. A 20-µl aliquot of the biotinylated PCR products was immobilized onto 3 µl streptavidin-coated Sepharose beads (Amersham Biosciences, Uppsala, Sweden) in 40 µl binding buffer, pH 7.6 (10 mM Tris-HCl, 2 mM NaCl, 1 mM EDTA, 0.1% Tween 20), in a 96-well plate. The plate was incubated at room temperature for 10 min with shaking (900 rpm) to keep the beads dispersed. Beads were harvested using a vacuum prep tool (Biotage AB, Uppsala Sweden); immersed for 5 s each in 70% ethanol, 0.2 M NaOH, and washing buffer at pH 7.6 (1 mM Tris-acetate); and dispensed into a 96-well plate containing 4 µl 10 mM primer in 40 µl annealing buffer, pH 7.6 (20 mM Tris, 2 mM magnesium acetate-tetrahydrate). The plate was heated on an 80°C heat block for 2 min to allow proper annealing. Pyrosequencing was performed using the reverse primer 5'-AGCAGTTGCAAATTGG in an automated PSQ96MA instrument using the PSQ 96 SNP reagent kit (Biotage AB, Uppsala, Sweden) according to the manufacturer's protocol.

Cloning and genetic manipulations. The *agaF* gene coding for EIIA^{Aga/Gam} from *E. coli* O157:H7 Sakai was amplified using 5' primer 5'-CAGTAAGCTT

FIG. 3. Growth of *E. coli* O157:H7 on M9 minimal agar plates with Aga or glucose (positive control). *E. coli* O157:H7, EDL933, and Sakai and two spinach isolates, EC4045 and EC4113, were streaked on M9 minimal medium agar plates with 0.2% glucose (A) and 20 mM Aga (B) as carbon sources and incubated at 37°C for 48 h.

ATGTTAAGTATTATTTTGACAGGGC-3' and 3' primer 5'-CAGTCTGCAG TCATATCCCTTCCTCGACCGGAC-3' (HindIII and PstI restriction sites are underlined). PCRs were carried out in 50-µl reaction mixtures as described above, and the PCR conditions were as follows: denaturation at 94°C for 10 min; followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min; and a final incubation at 72°C for 10 min. The amplicon was digested with HindIII and PstI and cloned into the HindIII and PstI sites downstream of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible *tac* promoter of the expression plasmid pJH118HE (7). The resultant plasmid, pJFagaF, was transformed by electroporation into six *E. coli* O157:H7 isolates derived from the 2006 outbreak and also into EDL933. The vector without the insert, pJF118HE, likewise was transformed separately into these same strains as controls.

Overexpression of wild-type EIIA^{Aga/Gam}. An *E. coli* O157:H7 isolate, EC4045, from the spinach-associated outbreak, transformed with either pJF118HE (for control experiments) or pJFagaF, was grown over night in LB broth with 100 µg/ml ampicillin with shaking at 37°C. The cultures were then diluted 200-fold with 40 ml of fresh LB broth with 100 µg/ml ampicillin and grown with shaking at 37°C until they reached an optical density at 590 nm (OD₅₉₀) of 0.3. Each culture was split into aliquots; one half was supplemented with IPTG to a final concentration of 0.5 mM, while the other half remained unsupplemented. The resultant four cultures were incubated at 37°C with shaking for 2 h. A 1-ml aliquot of each culture was pelleted by centrifugation; the bacterial pellets were resuspended in 100 µl of sodium dodecyl sulfate (SDS) sample buffer and placed in a boiling water bath for 3 min. A 5-µl aliquot of the whole-cell proteins was separated on 12.5% SDS-polyacrylamide gels and visualized by Coomassie blue R-250 staining.

RESULTS AND DISCUSSION

***E. coli* O157:H7 isolates derived from the 2006 spinach-associated outbreak cannot utilize Aga.** PMs are run routinely in this laboratory on bacterial strains that are to become part of our enteric reference collection. Such analyses uncovered phenotypic differences between *E. coli* O157:H7 isolates derived from the 2006 spinach-associated outbreak and those derived from diverse sources and contained within our reference collection. Whereas none (0/25) of the recent outbreak-derived isolates could utilize Aga, 98% (118/120) of the *E. coli* O157:H7 strains in the reference collection could utilize this amino sugar as either a primary carbon or nitrogen source. A typical PM plot using two strains, EDL933 as control and EC4045, a spinach isolate, exemplifies the Aga (as a carbon and nitrogen source) utilization differences in these two populations (Fig. 2). Homing in on this phenotypic difference, plating experiments were carried out on 194 isolates from the recent outbreak and 120 strains of *E. coli* O157:H7 from the reference collection. In Fig. 3, the results are shown for two of the isolates from the spinach-associated outbreak, EC4045 and EC4113, and for two control strains, EDL933 and Sakai. As

expected, 100% (314/314) of the isolates grew on glucose plates (as in Fig. 3A). When Aga was the sole carbon source, 98% (118/120) of the *E. coli* O157:H7 strains from the reference collection could utilize this amino sugar, whereas none (0/194) of the isolates from the 2006 outbreak could grow on Aga (as in Fig. 3B). Growth properties on M9 agar medium similar to those shown in Fig. 3B were observed for these two populations when Aga served as both carbon and nitrogen source (data not shown).

***E. coli* O157:H7 strains cannot utilize Gam.** As isolates from the 2006 spinach-associated outbreak were unable to utilize Aga and because the transport and catabolism of Aga and Gam are linked (3), the PM data for Gam utilization were also examined. It should be noted that the PM system incorporates Gam only as a nitrogen source (PM 3), not as a sole carbon source. The PM data indicated that none of the 120 *E. coli* O157:H7 isolates from the reference collection and none of 25 isolates from the spinach-associated outbreak utilized Gam (data not shown). In contrast, *E. coli* C, which is known to grow on Gam (3), metabolized this amino sugar efficiently (data not shown). This was surprising, as it had been reported earlier that *E. coli* O157:H7 could utilize both Aga and Gam (23). We thus sought to resolve whether differences between the previous and present reports might be due to the type of assay employed. The assay conditions are especially critical with Gam, as it is known that Gam, like D-glucosamine and D-mannosamine, undergoes nonenzymatic browning reactions, i.e., modifications at pH 7.0 in phosphate buffer (conditions that exist in M9 medium), that lead to decomposition, transformation, and formation of nondialyzable material suggestive of polymerization (8), which may affect growth. It is also known that N acetylation of the amino sugars prevents the browning reactions (8). In this laboratory, *E. coli* C failed to grow on M9 minimal medium agar plates with Gam as a carbon source, probably due to browning reactions. However, when *E. coli* C was incubated in liquid M9 medium containing Gam at 37°C with shaking for 48 h, it reached an OD₅₉₀ of 1.0 to 1.5, even though the medium turned slightly yellow over that time period. Under similar conditions, none of the 120 *E. coli* O157:H7 reference isolates grew on Gam in M9 minimal medium as measured by turbidity (OD₅₉₀ < 0.1). Thus, based on both PM results and the liquid incubation assay, we report a Gam⁻ phenotype for *E. coli* O157:H7, contrary to the earlier report (23); in silico analysis (see below) further supports this conclusion.

In silico analyses of the *aga/gam* cluster of genes in *E. coli* O157:H7 indicate its inability to utilize Gam. The *aga/gam* clusters of genes of two *E. coli* O157:H7 strains, EDL933 and Sakai, and *E. coli* C (3) were aligned (GenBank references for strains EDL933 and Sakai are NC_002655 and NC_002695, respectively). Annotation of this region is thorough for the EDL933 strain (relative to Sakai), and for this reason, relative comparisons are made between *E. coli* C and the EDL933 strain. As shown in Fig. 1A, 11 of the 13 genes in the *E. coli* C gene cluster are present in EDL933 (and Sakai), and these 11 genes from *E. coli* O157:H7 code for proteins with 98% homologies to those found in *E. coli* C. It should be noted that the Aga deacetylase encoded by *agaA* in *E. coli* C (ECs4015 in Sakai) is seven amino acids longer than its EDL933 homologue because the in silico translation is from an ATG codon 21 bp

upstream from the ATG codon in EDL933. More notable, however, are the differences revealed in the EDL933 *agaI* and *agaC* genes (encoding Gam-6-P-deaminase/isomerase and EIIC^{Gam}, respectively) compared with their *E. coli* C homologues. A split gene annotation is reported for EDL933 (but not Sakai) because a substitution within the 72nd codon (CAG, coding for glutamine) of the EDL933 (and Sakai) *agaI* gene results in a premature amber stop codon (TAG) being introduced. That is, in contrast to the case for the *E. coli* C *agaI*, which codes for a 251-amino-acid protein, the annotation for EDL933 catalogs *agaI* as a gene coding for a 71-amino-acid polypeptide and a second, 169-amino-acid protein, initiating in frame at the 83rd codon. Whether either of the transcripts and the corresponding protein products are made efficiently within the cell is speculative. Note, however, that the *agaI* gene product, Gam-6-P deaminase/isomerase, deaminates Gam-6-P and isomerizes D-galactose-6-P to tagatose-6-P. As most *E. coli* O157:H7 strains can utilize Aga as sole carbon and nitrogen sources and recalling the commonality of the Aga and Gam pathways after deacetylation of Aga-6-P to Gam-6-P, it is unlikely that the base substitution in the EDL933 *agaI* gene can account for the Gam⁻ phenotype found in *E. coli* O157:H7.

Although beyond the scope of the present studies, the question remains as to how Aga is utilized in *E. coli* O157:H7 when there is no functional Gam-6-P deaminase/isomerase. As no experimental evidence exists to date, two plausible explanations are suggested. The catabolic pathway for N-acetyl-D-glucosamine in *E. coli* is similar to the Aga pathway (3, 16). It has been reported that Gam-6-P deaminase/isomerase is a homologue of D-glucosamine-6-P deaminase/isomerase, with great similarity (28% identity) to the *E. coli* enzyme coded by *nagB* (20). Although the structure of *E. coli* D-glucosamine-6-P deaminase/isomerase has been solved (15) and its kinetic properties studied (4), its activity using Gam-6-P as a substrate has not been tested. However, another enzyme of the *E. coli* N-acetyl-D-glucosamine pathway, N-acetyl-D-glucosamine deacetylase, has been shown to have 10% activity with Aga as a substrate (22). Also, *E. coli* K-12 mutants have been isolated that have an Aga⁺ Gam⁻ phenotype and carry suppressor mutations in the genes for N-acetyl-D-glucosamine utilization and transport Aga by the N-acetyl-D-glucosamine PTS, after which the Aga is deacetylated by N-acetyl-D-glucosamine-6-P deacetylase encoded by *nagA* (3). Considering these facts, it is possible that D-glucosamine-6-P deaminase/isomerase could substitute for the lack of Gam-6-P deaminase/isomerase in *E. coli* O157:H7. Alternatively, it is possible that a known or unidentified gene outside this cluster might carry out the function of Gam-6-P deaminase/isomerase. Such an example exists for this pathway, where phosphofructokinase, which phosphorylates tagatose-6-P, is encoded by *pfkA*, which lies outside this gene cluster.

With regard to *agaC*, relative to the *E. coli* C sequence, a C:G→T:A substitution within the eighth codon of *agaC* in EDL933 (and Sakai) results in an amber stop codon (TAG) rather than the encoding of a glutamine residue (CAG). This leads to the EDL933 *agaC* gene encoding a truncated product. Rather than the 267-amino-acid protein encoded by the *E. coli* C *agaC* gene, the EDL933 gene, initiating in frame at the 77th codon (*E. coli* C sequence), codes for a 191-amino-acid protein. It should be noted that in *E. coli* O157:H7 Sakai, the two

genes *agaC* and *agaI* are not annotated. Studies on the membrane topology of EIIC^{Man} (ManY) of *E. coli* K-12 have shown that it has six membrane-spanning segments with the N and C termini facing the cytoplasm, and it has also been suggested that the first 21 residues may function as a signal sequence (9). Clustal W 1.83 alignment of EIIC^{Man} and EIIC^{Gam} reveals that there is significant sequence similarity between the two, with 65/266 identical residues and 78/266 conserved residues (data not shown), suggesting that EIIC^{Gam} may have a similar membrane topology. It is highly unlikely that EIIC^{Gam} with 76 amino acids truncated from the N-terminal end, which encompasses the leader sequence and the first two membrane-spanning segments (9), would integrate into the membrane. Thus, even if a truncated stable EIIC^{Gam} is produced in the cell, it would be inactive and render the cell defective in the uptake of Gam.

***E. coli* O157:H7 isolates from the spinach-associated outbreak have a point mutation in the *agaF* gene.** As none of the spinach-associated outbreak isolates grew on Aga, the nucleotide sequences of the region harboring the cluster of genes for transport and catabolism of Aga and Gam in two of these isolates (EC4042 and EC4191) were examined and compared with the known sequences of strains EDL933 and Sakai. Sequence alignment of the 11,745-bp *aga/gam* cluster of genes in EDL933 (coordinates 4084307 to 4095781) with those in the Sakai strain and isolates EC4042 and EC4191 revealed a single nucleotide difference (G:C→A:T) in the *agaF* gene coding for EIIC^{Aga/Gam} (annotated as Z4488 in EDL933 and as ECs4014 in Sakai) in both EC4042 and EC4191, leading to a Gly91Ser substitution. To ascertain the frequency of this mutation in the 194 isolates from the 2006 outbreak, pyrosequencing of this region was done. Pyrosequencing is an ideal method to sequence short stretches of DNA (10 to 100 bases) and is routinely used in this laboratory for single-nucleotide polymorphism analyses of bacterial genomes (5). Pyrosequencing of this region, as described in Materials and Methods and depicted in Fig. 4C, was carried out on the 194 isolates from the 2006 outbreak. The results from pyrosequencing showed that 100% (194/194) of the isolates harbored the same C→T transition mutation. Pyrograms of Sakai (Fig. 4A) and EC4001, an isolate from the spinach-associated outbreak (Fig. 4B), are shown to demonstrate the ready identification of this allelic difference within *agaF*. Of interest, the two isolates from the reference collection of *E. coli* O157:H7, namely, EC508 and EC1264, which did not grow on Aga also harbored the same mutation within the *agaF* gene (data not shown).

The wild-type *agaF* can complement the Aga⁻ phenotype in spinach outbreak isolates. To investigate whether the point mutation in *agaF* in these isolates was responsible for the Aga⁻ phenotype, complementation experiments using a wild-type copy of *agaF* were conducted. The construction of the plasmid, pJFagaF, used for complementation is described in Materials and Methods. Briefly, the *agaF* gene from the Sakai strain (annotated as ECs4014) was cloned into the expression vector pJF118HE downstream of the *tac* promoter such that the expression of *agaF* could be regulated by varying the IPTG concentration. To confirm that EIIC^{Aga/Gam} (AgaF) is overproduced from pJFagaF, this plasmid and pJF118HE as a control were each transformed into a spinach isolate, EC4045, harboring the *agaF* mutation. Although no band corresponding to the

FIG. 4. Typical pyrograms from pyrosequencing of the region covering the point mutation in the *agaF* gene in *E. coli* O157:H7. (A and B) Pyrograms of two *E. coli* O157:H7 strains, Sakai (A) and spinach isolate EC4001 (B). The ordinate in the pyrograms indicates light intensities in AU. The sequence from the Sakai strain (A), which can utilize Aga, is TGCCGGTG, whereas the sequence from strain EC4001 (B), which cannot utilize Aga, is TGCTGGTG. The peak heights are a quantitative measure of the nucleotide present; thus, double the peak height indicates the dinucleotide sequence CC in panel A and GG in panels A and B. The yellow regions indicate the region of the nucleotide difference between the Sakai strain and EC4001. (C) The sequencing primer (indicated by an arrow) and the sequence of the reverse strand that is sequenced. The codon for glycine is underlined, and the nucleotide change (G:C to A:T) that results in a serine codon in spinach isolates is indicated by bold and large lettering.

predicted size of EIIC^{Aga/Gam} (15.5 kDa) is visible in either the uninduced culture or the IPTG-induced culture of EC4045 with pJF118HE (Fig. 5, lanes 1 and 2), a distinct band, corresponding to EIIC^{Aga/Gam}, is seen in EC4045 cultures carrying pJFagaF upon induction with 0.5 mM IPTG for 2 h (Fig. 5, lane 4) but not in the uninduced cultures (Fig. 5, lane 3). This experiment demonstrates that EIIC^{Aga/Gam} is indeed expressed from pJFagaF and can be overexpressed in the transformed spinach isolate. Complementation experiments were performed by transforming pJFagaF into each of six independent *agaF* mutants harboring the G:C→A:T mutation and into EDL933 (as a control). Plasmid pJF118HE was similarly transformed into these same isolates for control experiments. Transformants were streaked onto M9 minimal medium agar plates containing 20 mM Aga as a carbon source and 100 µg/ml of ampicillin, either without IPTG or with IPTG at concentrations of 50, 100, and 500 µM. As a positive control, transformants were streaked onto M9 minimal medium agar plates with 0.2% glucose and 100 µg/ml of ampicillin. The results with all six isolates were the same, and the results for two of these mutants, isolates EC4045 and EC4143, are described here. All transformants, harboring either pJF118HE or pJFagaF, grew

FIG. 5. SDS-polyacrylamide gel electrophoresis of overexpression of wild-type EIIA^{Aga/Gam} in EC4045, a spinach isolate. Overexpression of EIIA^{Aga/Gam} and SDS-polyacrylamide gel electrophoresis are described in Materials and Methods. The samples in the lanes are EC4045 with pJF118HE (lanes 1 and 2) and EC4045 with pJFagaF (lanes 3 and 4). The uninduced (without IPTG) samples are in lanes 1 and 3, and the IPTG-induced samples are in lanes 2 and 4. Molecular weight (MW) markers were run in lane 5, and the molecular weights in thousands are shown next to each band. The overexpressed EIIA^{Aga/Gam} protein band in lane 4 is indicated by an arrow.

on the positive control glucose M9 minimal agar medium plates (Fig. 6A), and whereas EDL933 strain (*agaF*⁺) transformed with either pJF118HE or pJFagaF grew on Aga plates, isolates EC4045 and EC4143 containing the mutant *agaF* allele grew on Aga only when they harbored pJFagaF and not pJF118HE (Fig. 6B). Notably, basal expression of *agaF* from pJFagaF (without IPTG) was sufficient to complement the *Aga*⁻ phenotype in the spinach isolates (Fig. 6B). Furthermore, results with higher levels of expression of EIIA^{Aga/Gam} (induced with increasing concentrations of IPTG) in these transformants were the same as those in Fig. 6B, suggesting that the high levels were not detrimental for growth (data not shown). These experiments, clearly demonstrating that a wild-type copy of *agaF* effectively complements the *Aga*⁻ pheno-

type, implicate the Gly91Ser change in manifesting this phenotype.

The Gly91 residue in EIIA^{Aga/Gam} is conserved among the EIIs of the Man, Sor, and Fru family. The experiments presented in the previous section show that the Gly91Ser mutation is responsible for rendering the cells ineffective in utilizing Aga. As this residue was critical for functional EIIA^{Aga/Gam} activity, we asked whether the Gly91 residue was conserved throughout the EIIs of the Aga, Man, Sor, and Fru PTSs from divergent bacteria. The crystal structure of the *E. coli* EIIA domain of the IIAB subunit of EII^{Man} has been solved by Nunn and coworkers (14). In that study it was also shown, using sequence alignments, that this glycine residue was conserved in EIIA^{Man} of *E. coli* K-12, EIIA^{Fru} of *B. subtilis* (12), EIIA^{Sor} of *Klebsiella pneumoniae* (25), and EIIA^{Man} of *Vibrio furnissi* (2), although a specific role for this glycine residue was not assigned (14). As a large number of bacterial genome sequences are now available, a BLAST search of the EIIA^{Aga/Gam} of *E. coli* O157:H7 was done and the Clustal W (1.83) multiple sequence alignment program (24) was used to align selected sequences of EIIA^{Aga} from three bacteria, EIIA^{Man} and EIIAB^{Man} sequences from seven bacteria, EIIA^{Sor} from *K. pneumoniae*, and EIIA^{Fru} from *B. subtilis* and *Rhodospirillum rubrum* (Fig. 7). In *E. coli* K-12, *Salmonella enterica* serovar Typhimurium LT2, *Yersinia pestis* KIM, and *Clostridium perfringens* ATCC 13124 the EIIA and EIIB are in the same polypeptide and hence have longer sequences, but only the EIIA domains and a few residues of the N-terminal domain of EIIB are shown (Fig. 7). The 13 sequences showed a range of identity from 98% for EIIA^{Aga} of *Shigella flexneri* 2a to 26% for EIIA^{Man} of *Agrobacterium tumefaciens* C58. Despite this wide variation in sequence identity and the inclusion of divergent bacteria, the Clustal alignment shows that the Gly91 residue of EIIA^{Aga/Gam} of *E. coli* O157:H7 is invariant in these 14 sequences. This suggests that this Gly91 plays a critical role. The alignment also shows that the His10 residue of the EIIA subunit from the Man, Sor, and Fru PTS family, which is phosphorylated by phospho-HPr (6, 14), is also invariant (Fig. 7). The crystal structure of the EIIA domain of the Man PTS from *E. coli* revealed that interactions of loop 1/A of one subunit with helix A of the other subunit and isologous contacts between the 4/D loops of both subunits are involved in the formation of the interface of the dimerized protein (14).

FIG. 6. Complementation of *E. coli* O157:H7 spinach isolates for growth on Aga with a wild-type *agaF* gene. *E. coli* O157:H7 strains EDL933, EC4045, and EC4113 transformed with plasmid pJF118HE (parent vector) as a control or pJFagaF were streaked on M9 agar plates with ampicillin (100 μ g/ml) and either 0.2% glucose (A) or 20 mM Aga (B) as carbon sources and incubated at 37°C for 48 h.

<i>E. coli</i> K-12	-----MTIAIVIGTGGWAAEQLLKTAEMLLGEQENVGWIDFVPGE	40
<i>S. Typhimurium</i>	-----MTIAIVIGTGGWAAEQLLKTAEMLLGEQENVGWIDFVPGE	40
<i>Y. pestis</i>	-----MSIAIIIGTGGAAAEQLLKTAEMILGEQSNVAYIDFVPGE	40
<i>C. perfringens</i>	-----MVGIIILASGGFEKAGILQSGSMIFGEQENVKAVTLMPSE	39
<i>L. monocytogenes</i>	-----MKAIFVCTGGNAAKELIQSAEMICGKQENTSFVPFEVNE	39
<i>E. coli</i> O157:H7	-----MLSIILTGEGGFASGMEKAMKQILGEQSQFIAIDFPETS	39
<i>S. flexneri</i>	-----MLSIILTGEGGFASGMEKAMKQILGDQSQFIAIDFPETS	39
<i>A. hydrophila</i>	MQASSNPVPAPGRPFVAVKEQIMLGIIICGGGFASGMEQAMLQILGDQEAVIDFPEAS	60
<i>V. fischeri</i>	-----MIGIVVSGHINFATGVESAVKAIAGEQEQMEFVDFVESM	39
<i>S. pneumoniae</i>	-----MKIVLVGHHGFATGIYSSQLLIAGNQENVEAIDFVEGM	38
<i>C. crescentus</i>	-----MAGMGIPFEIQGMIGLVIVTGGRLAEFFVSAMEHVVGPPAAVKAICIGPED	51
<i>A. tumefaciens</i>	-----MIGLVLVTHGKLAEFRHALEHVVGPKLIETVCIGPED	39
<i>R. rubrum</i>	-----MIGMLVTHGRLAVELINATEHVMGKQENIAAVCIGPED	39
<i>B. subtilis</i>	-----MISVIISGHHDFPIALKESSGMIFGEENNLIAVPFFKGE	39
	: : * . . : : * : : :	
<i>E. coli</i> K-12	NAETLIEKYNAQLAKLDTT-KGVFLFVDTWGGSPFNAASRIVVD--KEHYEVIAGVNI	97
<i>S. Typhimurium</i>	NAETLIEKYNAQLAKLDTS-KGVFLFVDTWGGSPFNAASRIVVD--KERYEVIAGVNI	97
<i>Y. pestis</i>	NAETLIEKYNDRLTGLDTS-KGVFLFVDTWGGSPFNAASRIAID--KENYEVVTVNI	97
<i>C. perfringens</i>	GPDDIRAKMKEAIAFDNQ-DEVFLFVDLWGGTPFNQADSLVEEH-KDKWAIIVAGN	97
<i>L. monocytogenes</i>	SAENLQAKIASEVAKLDLT-EGVFLFVLDLGGTFFNVLRLLGN--FDAVELVAGVNI	96
<i>E. coli</i> O157:H7	STALLTSQLEEAIAQLDCE-DGIVFLTDLLGGTPFRVASTLAMQKP--GCEVITG	96
<i>S. flexneri</i>	SAALLTSQLEEAIAQLDCE-DGIVFLTDLLGGTFFRVASTLAMQKP--GCEVITG	96
<i>A. hydrophila</i>	TTALLQDQCETALTQVERG-DGVVFLTDLLGGTFFRVASTLALRQP--GREVVTG	117
<i>V. fischeri</i>	STDELEQALRAAAKRVDSG-EGVFLFVLDIPGGSPANRALTIILMDT--ENVELIG	96
<i>S. pneumoniae</i>	SADELKQKILLAIS---NE-EEVLILSDLLGGSPFKVSSTIMGENPAKTMNVLS	94
<i>C. crescentus</i>	DMERRRADIILKACAAVDEGGDGVILLTDMFGGTPSNLAISVMEQTK---aEVIAG	108
<i>A. tumefaciens</i>	DMDQRRQDIIDAVARANDG-NGVILTDMFGGTPSNLAISVMNNGN---VEVIAG	95
<i>R. rubrum</i>	DMEERRQDILRCIDKVDTG-DGTILLTDMFGGTPSNLAISVMEKAK---IEVIAG	95
<i>B. subtilis</i>	GIQTLQEKYHQALKDIPPEE-HEVFLFVDIFGGTPYNAASFIAEDQ--RMDMAAG	96
	. : * * * : * . . : * * :	
<i>E. coli</i> K-12	LVETLMARDDDPFDELVALAVETGREGVKALKAKPVEKAAPAPAAAAAP-KAAP	156
<i>S. Typhimurium</i>	LVETFMARDDDPFDELVALAVETGREGVKALKAKPVEKAAPAPVAAAP-KAA-AP	155
<i>Y. pestis</i>	LAETFMARDDDPFDELVALAVETGREGVKALKAPDVKSDDKPTQPAAPVPTAKAP	157
<i>C. perfringens</i>	VIBAYASRFSMESAQEIAANILKSAREGVKVK---PESLEPEESTKATTDQAQ	153
<i>L. monocytogenes</i>	LLEAFLSR-ETLSLQQLANQVAETGRLGIYEEY-----AAP-----	130
<i>E. coli</i> O157:H7	LLEMVLER-EGLSGGEFRVQALECGHRLTS--LVDELGRCHEECPVEEGI-----	144
<i>S. flexneri</i>	LLEMVLER-EGLSGGEFRVQALECGHRLTS--LVDELGRCHEECPVEEGI-----	144
<i>A. hydrophila</i>	LLEMVMER-GELTPSQFREQLCCGHRLTS--LVDELARERAEALAEEGI-----	165
<i>V. fischeri</i>	VANAAFER-DDTTVKELVTT-LLEIGSSCMQDMRLQLELFMEPEPECEDGL-----	145
<i>S. pneumoniae</i>	LMEAVFAR-MAHSFDEVVNSVVAAGGVVNGKELFSTDAEEEEEDFESGI-----	144
<i>C. crescentus</i>	LIKLASVR-QRETQACVAHAQEAGRKYISVASVVLAGEK-----	147
<i>A. tumefaciens</i>	LIKLAGVR-SEDNMDKALQDASDAGRKYINVASRVLSGK-----	133
<i>R. rubrum</i>	LIRLATVR-DTQSLQDAATSAQEAGRKYINVASRLLTQNP-----	134
<i>B. subtilis</i>	LLEVLSLR-EHLALKDLLNKLKMSQQSFQVCSHLEKVKNTANQDTREDEL-----	146
	: . *	

FIG. 7. Clustal W (1.83) alignment of EIIA^{Aga/Gam} (AgaF) of *E. coli* O157:H7 EDL933 with the EIIs of the Man, Sor, and Fru PTSs from 13 different bacteria. A BLAST search of EIIA^{Aga/Gam} of the EDL933 protein sequence was carried out; the EIIA protein sequences of the Man, Sor, and Fru PTSs from 13 different bacteria were selected; and alignment was carried out using Clustal W (1.83). The sequences are as follows: EIIA^{Man} domains of the EIIAB^{Man} proteins from *E. coli* K-12 (accession no. NP_416331.1), *Salmonella enterica* serovar Typhimurium LT2 (accession no. NP_462671.1), *Yersinia pestis* KIM (accession no. NP_669855.1), and *Clostridium perfringens* ATCC 13124 (accession no. YP_695269.1); EIIA^{Man} from *Listeria monocytogenes* F6900 (accession no. EBA33912.1); EIIA^{Aga} from *E. coli* O157:H7 EDL933 (accession no. AAG58266), *Shigella flexneri* 2a strain 2457T (accession no. NP_838644.1), *Aeromonas hydrophila* ATCC 7966 (accession no. YP_855350.1), and *Vibrio fischeri* ES114 (accession no. YP_206958.1); EIIA^{Man} from *Streptococcus pneumoniae* D39 (accession no. YP_815782.1), *Caulobacter crescentus* CB15 (accession no. NP_419059.1), and *Agrobacterium tumefaciens* C58 (accession no. NP_353070.1); EIIA^{Fru} from *Rhodospirillum rubrum* ATCC 11170 (accession no. YP_428528.1); and EIIA^{Fru} from *Bacillus subtilis* 168 (accession no. NP_390585.1). The EIIAB proteins from *E. coli* K-12, *S. enterica* serovar Typhimurium LT2, *Y. pestis* KIM, and *C. perfringens* ATCC13124 have 324, 322, 323, and 326 amino acid residues, respectively. The EIIA^{Man} domain in EIIAB of *E. coli* K-12 is the first 133 residues, and the two domains are separated by an alanine- and proline-rich linker (14). The conserved Gly91 and His10 residues are indicated in boxes. Asterisks, colons, and periods indicate that amino acid residues are identical, conserved substitutions, and semiconserved substitutions, respectively.

That study also showed that the interface consists primarily of apolar residues. Interestingly, the Gly92 residue of EIIA^{Man} of *E. coli* K-12, which is the Gly91 residue of EIIA^{Aga/Gam} in *E. coli* O157:H7, lies in the 4/D loop. Thus, a plausible explanation for the inactivation of EIIA^{Aga/Gam} by the Gly91Ser mutation would be that the replacement of apolar glycine in the 4/D loop with a polar serine affects dimer formation and hence its function.

Concluding remarks. Most interesting in the present studies is the Aga⁻ phenotype uncovered in the isolates derived from the 2006 spinach-associated outbreak of *E. coli* O157:H7. Clearly, it presents as a biomarker that can be exploited by microbiological or molecular means to differentiate these isolates from the majority of *E. coli* O157:H7 isolates described thus far. This phenotype may be telling us more. For example, as a follow-up to this study, we have also found this same

mutation in *E. coli* O157:H7 isolates derived from more recent produce-associated outbreaks (data not shown). Such findings suggest the radiation of this *agaF* mutation (and thus the Aga[−] phenotype) through the *E. coli* O157:H7 population. At present, we can only speculate whether this phenotype represents the recent radiation of a neutral mutation throughout the population or, more intriguingly, the result of the adaptation of *E. coli* O157:H7 to newer niches; i.e., the Aga[−] phenotype may indeed confer a selective survival advantage to *E. coli* O157:H7 in its new environment.

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