More than Rotating Flagella: Lipopolysaccharide as a Secondary Receptor for Flagellotropic Phage 7-7-1

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ABSTRACT Bacteriophage 7-7-1, a member of the family Myoviridae, infects the soil bacterium Agrobacterium sp. strain H13-3. Infection requires attachment to actively rotating bacterial flagellar filaments, with flagellar number, length, and rotation speed being important determinants for infection efficiency. To identify the secondary receptor(s) on the cell surface, we isolated motile, phage-resistant Agrobacterium sp. H13-3 transposon mutants. Transposon insertion sites were pinpointed using arbitrary primed PCR and bioinformatics analyses. Three genes were recognized, whose corresponding proteins had the following computationally predicted functions: AGROH133_07337, a glycosyltransferase; AGROH133_13050, a UDP-glucose 4-epimerase; and AGROH133_08824, an integral cytoplasmic membrane protein. The first two gene products are part of the lipopolysaccharide (LPS) synthesis pathway, while the last is predicted to be a relatively small (13.4-kDa) cytosolic membrane protein with up to four transmembrane helices. The phenotypes of the transposon mutants were verified by complementation and site-directed mutagenesis. Additional characterization of motile, phage-resistant mutants is also described. Given these findings, we propose a model for Agrobacterium sp. H13-3 infection by bacteriophage 7-7-1 where the phage initially attaches to the flagellar filament and is propelled down toward the cell surface by clockwise flagellar rotation. The phage then attaches to and degrades the LPS to reach the outer membrane and ejects its DNA into the host using its syringe-like contractile tail. We hypothesize that the integral membrane protein plays an important role in events following viral DNA ejection or in LPS processing and/or deployment. The proposed two-step attachment mechanism may be conserved among other flagellotropic phages infecting Gram-negative bacteria.

IMPORTANCE Flagellotropic bacteriophages belong to the tailed-phage order Caudovirales, the most abundant phages in the virome. While it is known that these viruses adhere to the bacterial flagellum and use flagellar rotation to reach the cell surface, their infection mechanisms are poorly understood. Characterizing flagellotropic-phage-host interactions is crucial to understanding how microbial communities are shaped. Using a transposon mutagenesis approach combined with a screen for motile, phage-resistant mutants, we identified lipopolysaccharides as the secondary cell surface receptor for phage 7-7-1. This is the first cell surface receptor identified for flagellotropic phages. One hypothetical membrane protein was also recognized as essential for infection. These new findings, together with previous results, culminated in an infection model for phage 7-7-1.

KEYWORDS complex flagellar filaments, glucose epimerase, glycosyltransferase, flagellar motor, motility, glycosyltransferase

Bacteriophages are part of all microbial ecosystems and thus influence evolution and population dynamics (1, 2). Estimates have placed the total global phage count at roughly 1031 phage particles, making them the most abundant organisms on Earth (3,
Rhizobium lupini H13-3 (39), is classified as a nonpathogenic member of the genus such and membrane proteins (10–12). Many large DNA phages of the order Podoviridae or Siphoviridae through their tail spikes (Podoviridae) or tail fibers (Siphoviridae and Myoviridae), located at the end of the phage tail (13). This first attachment to LPS is specific but reversible. The tail-associated complexes possess receptor-destroying enzymatic activity, resulting in the cleavage of LPS O-antigen repeats. The specificity of tail spike-associated hydrolases is a determinant of host range. Degradation of LPS allows the phage to pass through the outer LPS region, gaining access to the secondary receptor on the cell surface or directly to the membrane. The second interaction results in irreversible attachment. Myoviridae phages, like T4, possess long, contractile tails that pierce the outer membrane of the host for ejection of viral DNA (13). The membrane-puncturing device disrupts the intermembrane peptidoglycan layer via lysozyme activity. The phage tail can directly penetrate the cytoplasmic membrane to eject its DNA or, alternatively, interacts with a bacterial cytoplasmic inner membrane protein to transfer phage DNA into the cytoplasm (14–17). Phages with short tails, like the podovirus P22, are not able to use their tails to reach the inner membrane. Instead they rely on interactions with secondary receptors on the cell surface to trigger release of viral proteins before genome ejection, which are hypothesized to aid in protection and transport of the DNA to and through the inner membrane. The process of DNA translocation is the least understood step in bacteriophage infection (9, 18–20).

Bacterial pili and flagella are targets of phages in the order Caudovirales (21). Flagellotropic phages, which use the bacterial flagellum as a host receptor for attachment, remain poorly characterized. The mere presence of flagella is not sufficient for infection. Flagellar rotation has been shown to be a requirement for infection, resulting in the proposal of a “nut and bolt” mechanism for phage translocation along the flagellar filament to the cell surface (21, 22). The family of flagellotropic phages is represented by $\chi$ of Escherichia coli and Serratia marcescens, φCB13 and φCbbK of Caulobacter crescentus, PBS1 and SP3 of Bacillus subtilis, φAT1 of Erwinia carotovora, F341 of Campylobacter jejuni, φ OT8 of Serratia sp. strain ATCC 39006 and Pantoea agglomerans, and 7-7-1 of Agrobacterium sp. strain H13-3 (21, 23–32). It is speculated that infection by flagellotropic phages is species specific due to the unique surface structures of bacterial flagellar filaments. For example, phage 7-7-1 infects only Agrobacterium sp. H13-3 and not closely related Rhizobiales species (33).

The lytic phage 7-7-1 was initially isolated from a compost soil in Germany (34) and is a member of the family Myoviridae. Members of this phage family consist of a head, a collar, and a contractile tail, with E. coli phage T4 the best-studied example (35). Phage 7-7-1 shows relatively little overall DNA sequence similarity to other phages (36). It has a hexagonal head with a diameter of 68 nm and a 135-nm-long contractile tail that is connected to bushy tail fibers (23, 34, 37). Its eclipse period is about 60 min, and phage propagation is completed after 80 min, with a burst size of 120 particles per bacterial cell (23, 38). The host of phage 7-7-1, Agrobacterium sp. H13-3, formerly known as Rhizobium lupini H13-3 (39), is classified as a nonpathogenic member of the genus Agrobacterium based on genome structure and phylogenetic analyses (40). The closely related nonhost Agrobacterium tumefaciens is well known as the causative agent for...
crown gall induction in plants, which places an economic burden on orchards and vineyards worldwide (41).

The clockwise-rotating *Agrobacterium* sp. H13-3 flagellum is classified as complex and consists of three flagellin proteins: the primary flagellin FlaA, which is essential for motility, and the secondary flagellins FlaB and FlaD, which have minor functions in motility (42). The role of individual flagellins in flagellar structure and motility is paralleled by their importance for phage infection. The absence of minor flagellins does not affect, or only moderately affects, infection, while lack of the major flagellin FlaA severely reduces infection efficiency. A mutant lacking all three flagellin genes is phage resistant (33). The speed of flagellar rotation also correlates with the efficiency of phage infection; a mutant with faster-rotating flagella is infected more effectively, while slower flagellar rotation significantly reduces the efficiency of infection. Furthermore, strains that are nonmotile are resistant to phage infection (33).

We hypothesize that phage 7-7-1 interacts with bacterial cell surface receptors after it has traversed down the flagellar filament and reached the cell surface. However, such cell surface receptors have not yet been identified. In this study, we used transposon mutagenesis combined with a screen for the motile, phage-resistant *Agrobacterium* sp. H13-3 mutants and investigated genetic variations underlying resistance. Bioinformatics analysis of transposon insertion sites found that two genes related to lipopolysaccharide biosynthesis, along with an integral inner membrane protein with unknown function, are essential for infection by phage 7-7-1. These new findings, together with previous results, culminated in the development of an infection model for phage 7-7-1.

**RESULTS**

Transposon mutagenesis of *Agrobacterium* sp. H13-3 and identification of phage-resistant, motile transposon mutants. We hypothesized that phage 7-7-1 infection required binding to secondary receptors on the cell surface of *Agrobacterium* sp. H13-3 following interactions with the primary receptors, flagella. We therefore developed a scheme to identify *Agrobacterium* sp. H13-3 mutants that are both phage resistant and motile. First, we created a pool of mutants by delivering a Tn5 transposon to RU12/001 via conjugation. A liquid tryptone-yeast extract-CaCl₂ (TYC) culture containing streptomycin and neomycin (selecting for *Agrobacterium* sp. H13-3 mutants containing a transposon insertion) was inoculated from the conjugation pool and grown until the cells became motile. Next, phage-resistant mutants in the culture were selected by continuous growth in the presence of phage. Transposon-containing *Agrobacterium* sp. H13-3 cells were further selected by plating on TYC agar plates with streptomycin and neomycin. To screen for motile, phage-resistant mutants, 1,000 colonies were spotted on Bromfield swim plates containing 0.3% Bacto agar (88). Since more than 40 genes are required to produce a functional flagellum (40, 43), the vast majority of the mutants were nonmotile. However, we identified 17 motile, phage-resistant mutants in this screen (Table 1). Using arbitrary primed PCR, we obtained flanking sequences from all the transposon insertion sites in our mutant set. The insertions resided in two genes, AGROH133_07337 (07337), annotated as a glycosyltransferase gene, and AGROH133_08824 (08824), whose gene product is predicted to be an integral membrane protein (Table 2). For 07337, eight insertion mutants were identified with six different insertion sites, while for 08824, nine insertion mutants with five different insertion sites were obtained. A second round of screening on swim plates was performed, and 16 motile mutants were identified out of a total of 800. Of these mutants, seven were 07337 insertional mutants, eight were 08824 mutants, and one mutant, PR18, had a transposon insertion in AGROH133_13050, coding for a UDP-glucose 4-epimerase (Table 2). In conclusion, we identified three *Agrobacterium* sp. H13-3 genes potentially important for mediating sensitivity to phage 7-7-1.

Verification of the phage-resistant phenotype by complementation and in-frame deletions. To verify that disruption of these three genes by the transposon mediated phage resistance, we performed two independent experiments. First, we chose P13 and P222 as representative transposon insertion mutants of genes 07337
TABLE 1 Bacterial strains and plasmids

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<td>S17-1</td>
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<td>Ap’ Km’ Tn5-110; transposon delivery vector</td>
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<td>Cm’ colE1 orl’ RP4tra+ RP4oriT; helper in triparental matings</td>
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aNomenclature according to Bachmann (96) and Novick et al. (97). The numbers in parentheses are the nucleotide positions of transposon insertions. Sm, streptomycin; Tc, tetracycline; Km, kanamycin; Ap, ampicillin; Cm, chloramphenicol; Tp, trimethoprim.

and 08824, respectively, along with PR18, the 13050 transposon mutant, and conjugationally transferred derivatives of the broad-host-range plasmid pBBR1MCS-3 (44) expressing the respective genes to generate complemented strains (Table 1). Second, we constructed unmarked in-frame-deletion strains for each gene by allelic replacement (Table 1). Next, we analyzed whether the resulting strains either restored sensitivity to phage (complemented strains) or gained resistance to phage (deletion strains). We performed infectivity assays by adding phage at a multiplicity of infection (MOI) of 1.0 to motile Agrobacterium sp. H13-3 NY cultures and allowing growth for 24 h before the optical density at 600 nm (OD600) was measured. Figure 1 displays the results for all the strains in comparison to the wild-type strain (RU12/001) and a nonflagellated mutant (RU12/006). Wild-type cultures in the absence of phage grew to an average OD600 of 1.80, but no growth was observed in the presence of phage. Growth of the nonflagellated strain, the transposon mutants, and the strains with 07337 (RU12/015), 08824 (RU12/016), and 13050 (RU12/017) deleted was unaffected by the addition of
phage. In contrast, transposon insertion strains carrying complementation plasmids behaved like the wild type and displayed no growth in the presence of phage. Transposon mutant PR18 (with a transposon insertion in the epimerase gene) grew to a statistically significantly lower OD$_{600}$ than the wild type ($P < 0.05$), but growth of the corresponding in-frame-deletion strain, RU12/017, was indistinguishable from that of the wild type. Therefore, the absence of gene 13050 was not the cause of the slight growth defect but possibly a secondary mutation or polar effect of the transposon

<table>
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<th>Gene and mutant</th>
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<th>Tn5 insertion site in gene (bp)</th>
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**FIG 1** Optical densities of cultures grown for 24 h at 30°C in the absence and presence of phage 7-7-1. Motile cultures were adjusted to an OD$_{600}$ of 0.03, and phage 7-7-1 was added at an MOI of 1.0. The black and gray bars depict cultures grown in the absence and presence of phage, respectively. Each bar represents the average from four independent experiments, and the error bars represent standard deviations (Student’s t test for RU12/001 and PR18; $P < 0.05$).
In summary, this portion of our work verified that the disruption of genes 03773, 08824, and 13050 caused the phage-resistant phenotype.

Motility of complemented transposon mutants and deletion strains. All the phage-resistant mutants of *Agrobacterium* sp. H13-3 described so far in the literature are nonmotile (23, 33). To demonstrate that motility does not contribute to the phage resistance of the identified transposon mutants, we quantified the motility of representative strains on Bromfield swim plates. After 4 days of incubation at 30°C, the wild type (RU12/001) formed a swim ring with a diameter of approximately 55 mm, whereas the nonmotile strain RU12/006 grew only at the inoculation point (Fig. 2). The swim ring diameters of transposon insertion and deletion strains were indistinguishable from that of the wild type, supporting the claim that motility is not a factor in conveying the phage resistance of strains defective in genes 03773, 08824, and 13050.

Putative functions of AGROH133_07337, AGROH133_13050, and AGROH133_08824. AGROH133_07337 codes for a glycosyltransferase (PF01755) belonging to family 25 (GT25) (45). This family of glycosyltransferases is populated by proteins that are solely involved in LPS biosynthesis, catalyzing the transfer of various sugar moieties from activated donor molecules to specific acceptors on the LPS chain during its biosynthesis (46).

AGROH133_13050 encodes a UDP-glucose 4-epimerase (EC 5.1.3.2), which carries out the reversible epimerization of UDP-glucose to UDP-galactose, the cognate substrate for galactosyltransferases. In analogy to orthologs from other Gram-negative bacteria, this gene can be named *galE* (47–49) and may also be directly involved in LPS biosynthesis.

AGROH133_08824 codes for a 128-amino-acid (aa) hypothetical protein, which is predicted to be an integral cytoplasmic membrane protein with up to four transmembrane helices (see Fig. S1 in the supplemental material) (50). The gene appears to be monocistronic and is located upstream of a gene cluster encoding the five subunits of an F-type proton-transporting ATPase (40). The gene synteny is conserved in all sequenced bacteria that carry the gene (40, 51–57).

Characterization of cell surface profiles of deletion strains. Since two of the three identified genes encode proteins involved in LPS synthesis, we examined the effects of their deletions on various cell surface properties. First, we purified LPS from the deletion and wild-type strains and analyzed their compositions by SDS-PAGE and silver staining. However, we were unable to identify differences in LPS composition
between the deletion mutants and the wild type (see Fig. S2 in the supplemental material). Next, we evaluated the effects of gene deletions on sensitivity to sodium chloride, a nonionic detergent (Triton X-100), and a bile acid (cholic acid), because changes in the LPS profile can result in increased sensitivity to these compounds (58, 59). In these assays, serial dilutions of the wild type and each deletion strain were spotted on tryptone-yeast extract (TY) plates supplemented with 3 M NaCl or TYC plates containing 0.5% Triton X-100 or 0.5% cholic acid. After incubation for 2 days at 30°C, we were unable to see significant differences between the control and experimental plates regardless of the treatment (see Fig. S3 in the supplemental material). We next assessed possible differences in polysaccharide profiles by spotting the deletion and wild-type strains on TYC plates containing Congo red or calcofluor white (60, 61). After 4 days of incubation at 30°C, slight differences between the deletion and wild-type strains were apparent on both types of plates. On plates containing calcofluor white, RU12/015 and RU12/016 fluoresced at intensities similar to those of the wild-type strain. Meanwhile, RU12/017 appeared duller (Fig. 3, top), indicating that the approximate numbers of (1→3)- and (1→4)β-glucopyranosyl units in the polysaccharide of RU12/017 were different (62). On Congo red-containing plates, RU12/017 clearly had increased pigment uptake, while pigment uptake in RU12/015 and RU12/016 appeared to be slightly above that of the wild-type strain but less than that of the above-mentioned mutant strains (Fig. 3, bottom). This result is suggestive of enhanced incorporation of (1→4)α-glucopyranosyl units into the polysaccharides of the deletion mutants, with the most significant increase in RU12/017 (62). It is important to note that in both assays, RU12/017 exhibited a rough colony phenotype while RU12/015 and RU12/016 were just as mucoid in appearance as the wild type. Nonetheless, the results demonstrate that the dye-binding properties and hence extracellular matrices (60) of all three deletion strains are different than that of RU12/001.

As a final means to characterize the surface properties of the four strains, the LPS isolates from the deletion and wild-type strains were submitted to matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (63). While the wild-type strain exhibited a repetitive set of ions with a Δm/z of 176, which is generally attributed to hexuronic acids or methylhexoses, the deletion mutants did not (Fig. 4). Since the full structures of LPS from Agrobacterium sp. H13-3, as well as the ionization efficiencies of the LPS isolates, are not known, it is not possible to assign structures to the observed spectra. However, it is relatively clear that the deletion strains are more similar to one another than to the wild-type strain.

**Adsorption of phage 7-7-1 to deletion strains.** As an indirect measure of alteration of cell surface structures, we quantified the adsorption of phage 7-7-1 to the wild-type and deletion strains. Phage 7-7-1 was added at an MOI of 0.01 to motile Agrobacterium sp. H13-3 cultures and incubated for 10 min to allow adsorption to occur. An equivalent volume of medium was mock treated with phage. Cell culture supernatants were assayed for PFU using plaque overlay assays, and the percentage of adsorption was calculated by normalizing to the medium control. There were no differences in adsorption of phage 7-7-1 to the wild type or the deletion mutant strains.
(Fig. 5). Although the average adsorption value for RU12/016 was lower than that for the other deletion strains, the result was not statistically significant ($P > 0.05$). The percent adsorption of phage to a strain that has paralyzed flagella, RU12/012, was the lowest of all the tested strains (Fig. 5), indicating that the mere presence of flagella is not sufficient to mediate the levels of adsorption seen with the wild type. We conclude that adsorption of phage 7-7-1 is not affected by the differences in cell surface structures of RU12/015, RU12/016, and RU12/017 but is affected by lack of motility or absence of flagella.

**DISCUSSION**

The recognition of specific host surface receptors is essential for successful bacteriophage infection. Flagellotropic phages use the bacterial flagellum for initial binding
and use its rotation to traverse down to the cell surface near the distal end of the flagellar filament (21, 22, 27), but further steps in the infection mechanism are poorly understood. The *C. crescentus* phages ΦCB13 and ΦCbK first make reversible contact with the bacterial flagellum before they irreversibly bind to the secondary surface receptor, the pilus portal. A rotating *C. crescentus* flagellum is not a prerequisite for infection, but it facilitates the positioning of phages in proximity to the pilus insertion sites and thus increases the probability of interaction (21). In contrast, a rotating bacterial flagellum is an absolute requirement for infection of *Agrobacterium* sp. H13-3 by bacteriophage 7-7-1 (33). However, the nature of a secondary phage receptor(s) on the *Agrobacterium* sp. H13-3 cell surface that mediates irreversible binding to the host is unknown.

In this study, Tn5 insertion mutants of *Agrobacterium* sp. H13-3 were selected for resistance to bacteriophage 7-7-1. Most of these mutants were nonmotile, confirming that motility is essential for infection. However, approximately 2% of the phage-resistant mutants were motile and exhibited wild-type behavior on swim plates (Fig. 2). Insertions were located in three genes, AGROH133_07337, AGROH133_08824, and AGROH133_13050 (Table 2), with AGROH133_07337 and AGROH133_13050 encoding enzymes that are part of the LPS-biosynthetic pathway. Although more than 50 genes are involved in LPS synthesis (64), we identified only two genes in our transposon mutagenesis screen that function in this role. This may be attributed to the essential nature of genes involved in the early biosynthetic steps and the deleterious effects of some mutations that affect the later steps (64). Furthermore, there is extensive evidence linking LPS mutations in other Gram-negative bacteria to defects in motility or complete abolishment of motility. These pleiotropic effects have been reported for *E. coli*, *Salmonella enterica* serovar Typhimurium, *Mesorhizobium loti*, *Rhizobium leguminosarum*, and *Rhizobium tropici* (65–69). Modifications to the LPS core, especially the inner core, have been shown to reduce motility, alter production of flagella, or eliminate flagellar synthesis in *E. coli* and *S. Typhimurium* (67, 69, 70). It is important to emphasize that infection of *E. coli* by the flagellotropic phage Χ is affected by LPS mutations because of the effects these mutations have on motility (71). Therefore, using motility as a selection mechanism, we could identify only mutants with LPS deficiencies that do not affect motility.

There were few discernible alterations between the LPS from the wild type and that of the deletion mutants as analyzed via silver staining after SDS-polyacrylamide gel electrophoresis (see Fig. S2 in the supplemental material). Additionally, the mutants were just as sensitive as the wild type to 0.3 M NaCl, 0.5% Triton X-100, and 0.5% cholic acid supplementing rich-medium agar plates (see Fig. S3 in the supplemental material), which goes against distinctive features of LPS mutants described in the literature (58, 59). A possible explanation for this observation is that these LPS modifications do not result in increased permeability to the compounds.

The subtle differences in polysaccharide profiles illustrated by Congo red and calcofluor white staining (Fig. 3) alongside unchanged phage 7-7-1 adsorption values for the wild-type and deletion strains (Fig. 5) provide ample evidence that the extracellular region of the mutants can still promote phage binding. These results initially led us to hypothesize that the modifications to LPS were minor. However, using a MALDI-TOF protocol targeting the lipid A component, we observed a clear difference between LPS isolates from the wild-type and mutant strains, with loss of an apparent repeat structure (Fig. 4). Adsorption to these mutant strains remained the same as that to the wild type (Fig. 5), despite the loss of the repeat structure, probably because motility remained unaffected (Fig. 2). Lower levels of phage 7-7-1 adsorption occurred only when binding to strains lacking flagella (RU12/006) or with paralyzed flagella (RU12/012) was evaluated (Fig. 5). Similar levels of adsorption to strains with paralyzed flagella have been documented for the flagellotropic phage Χ (25). This suggests that motility is important, not only for infection by flagellum-targeting phages but also for adsorption to their bacterial hosts. It is worth mentioning that strains PR18 and RU12/017 lacking the epimerase both had drier colony morphologies than the other strains,
which became more evident after storage of plates at 4°C, indicating involvement of
the 13050 gene product in extracellular polysaccharide (EPS) production. This is highly
plausible considering that the epimerase reaction occurs well before bifurcation of the
LPS and EPS pathways. This observation is also consistent with the results gained from
spotting the strains on Congo red- and calcofluor white-containing TYC plates (Fig. 3).

Although the structure of Agrobacterium sp. H13-3 LPS is unknown, closely related
Agrobacterium and Rhizobiaceae species use galactose as a building block (72). Hence,
13050 (galE) may provide the activated galactose moiety for use by the GT25 glyco-
syltransferase (AGROH133_07337). The O-specific polysaccharide (antigen) represents
the most variable part of the LPS, partially due to the large monosaccharide variety,
which results in enormous structural diversity. Since changes in LPS due to loss of
AGROH133_07337 or AGROH133_13050 function caused phage resistance, we con-
cludethataspecificregionofLPScontainsthephage receptor. Our resultssuggest that
galactose is an important part of the phage receptor structure. This conclusion is
supported by the fact that the E. coli C lysing phage ΦX174 does not adsorb to LPS
lacking the core terminal galactose residue (73). In addition to the primary flagellar
receptor mediating host specificity, the variability in the LPS structure of Rhizobiaceae
adds an extra layer of specificity for phage 7-7-1 adsorption. This may help explain the
narrow host range of 7-7-1, which is restricted to Agrobacterium sp. H13-3 (33). The
exactstructure of the LPS receptor unit remains to be elucidated.

The third gene identified as essential for Agrobacterium sp. H13-3 infection by phage
7-7-1, AGROH133_08824, encodes a small, hypothetical integral cytoplasmic membrane
protein consisting of up to four transmembrane helices. The function of the gene
product in infection is unknown, but we hypothesize that it is involved in events
following ejection of viral DNA into the host cell cytoplasm or in a step during LPS
biosynthesis and transport from the inner membrane to the outer membrane. LPS
assembly and translocation are intricate ATP-dependent processes. In E. coli and
Salmonella Typhimurium, an inner-membrane-spanning protein, MsbA, and the
LptB,FGC protein complex play central roles in LPS transport that are energized by ATP
hydrolysis (74–76). MsbA flips newly synthesized LPS from the cytoplasm to the
periplasmic inner membrane leaflet, where LPS interacts with LptB,FGC (59). Binding of
LPS by LptC initiates its transport along the LptCAD protein bridge connecting the
inner and outer membranes (77). At the end of the protein bridge, the translocon LptDE
exports LPS and inserts it in the outer membrane (60). AGROH133_08824 may serve as
an accessory protein to one of the two processes at the inner membrane executed by
MsbA or LptB,FGC. Interestingly, the AGROH133_08824 gene is located upstream of
genes predicted to code for the five subunits of an F-type proton-transporting ATPase.
Both the presence of this gene and the gene synteny are conserved in various
Agrobacterium and Rhizobium species (see Fig. S1 in the supplemental material),
indicating that the function of AGROH133_08824 may be important in these systems.
Investigation of the role of this gene in phage infection will be a subject of future
studies.

We created the working model depicted in Fig. 6 by drawing from our current and
pastfindings on phage 7-7-1 infection of Agrobacterium sp. H13-3. To paint a more
complete picture, we also utilized findings from studies centered around LPS-targeting
phages and phages that use inner membrane proteins during infection to further
inform our model. We propose the following. To initiate infection, phage 7-7-1 attaches
to the flagellar filament and uses its clockwise rotation to move toward the cell surface
(Fig. 6A). Next, the phage’s tail fibers bind to the secondary receptor, which in this
system is likely a particular region of the O-specific polysaccharide-containing galac-
tose. As has been shown for other phages that utilize LPS as a receptor, phage 7-7-1
enzymatically cleaves specific sugars on the polysaccharide chain after binding,
resulting in access to the outer membrane. The degrading enzyme is likely located at
the phage tail end, as was first described for P22 (78, 79). Subsequently, the phage tail
punctures the outer membrane and degrades the peptidoglycan layer; this
peptidoglycan-degrading activity has been demonstrated in other phages (35, 79, 80).
The viral DNA is then ejected into the host cell (Fig. 6B). We currently have two hypotheses regarding the role of the inner membrane protein in phage 7-7-1 infection. The protein may interact with the phage tail to trigger its anchoring into the host’s inner and outer membranes prior to ejection of viral DNA or in direct viral DNA transport, as has been demonstrated in other systems (14–17) (Fig. 6C1). Alternatively, the integral membrane protein could function in some part of LPS assembly and transport to the outer membrane (Fig. 6C2). It remains to be seen if the host specificity of phage 7-7-1 is dictated by these outer membrane modifications. Moreover, future studies will investigate whether other flagellotropic phages, such as the *E. coli* phage H9273, use LPS as a secondary receptor.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** Derivatives of *E. coli* K-12 and *Agrobacterium* sp. H13-3 (81) strains and the plasmids used are listed in Table 1.

**Media and growth conditions.** *E. coli* strains were grown in LB medium at 37°C (82). *Agrobacterium* sp. H13-3 was grown in TYC (0.5% tryptone, 0.3% yeast extract, 0.087% CaCl₂·2H₂O [pH 7.0]) or NY (0.8% nutrient broth, 0.3% yeast extract) medium, as indicated, at 30°C (83). The following antibiotics were used at the indicated final concentrations in LB, NY, or TYC medium: for *E. coli*, ampicillin at 100 μg ml⁻¹, chloramphenicol at 30 μg ml⁻¹, kanamycin at 50 μg ml⁻¹, and tetracycline at 10 μg ml⁻¹; for *Agrobacterium* sp. H13-3 in TYC medium, neomycin at 120 μg ml⁻¹ (12 μg ml⁻¹ in liquid medium), tetracycline at 10 μg ml⁻¹ (2.5 μg ml⁻¹ in liquid medium), and streptomycin at 600 μg ml⁻¹.

**Phage preparation.** *Agrobacterium* sp. H13-3 was grown in NY at 30°C to an OD₆₀₀ of 0.6. After ensuring that the bacteria were motile, the culture was diluted in 200 ml NY medium to a final OD₆₀₀ of 0.03. The bacteria were infected with phage 7-7-1 at an MOI of 0.005 and shaken for 24 h at 30°C. The resulting phage-containing lysate was adjusted to 4% NaCl, left on ice for 30 min, and centrifuged at 10,000 × g for 30 min at 4°C. Polyethylene glycol 8000 was added to the supernatant, resulting in a final concentration of 10% (wt/vol), and incubated for 16 h at 4°C. Phage particles were sedimented at 15,000 × g for 30 min at 4°C and suspended in 2 ml TM buffer (20 mM Tris-HCl [pH 7.5], 10 mM MgSO₄). The concentrated phage suspension was then layered on a 10 to 50% (wt/vol) iodoxanol (OptiPrep; Accurate Chemical and Scientific Corporation, Westbury, NY) gradient and centrifuged at 200,000 × g for 2 h at 15°C, using an SW-41 Ti rotor. A visible band approximately 20 mm from the bottom of the gradient was extracted with an 18-gauge syringe and dialyzed against TM buffer at 4°C with two buffer...
changes. Following this protocol, the phage titer ranged from 10^11 to 10^12 PFU ml⁻¹. The final phage stock was stored in TM buffer at 4°C.

**Infectivity assays.** Cultures were grown in NY medium from single colonies at 30°C for 18 to 26 h until they reached an OD₆₀₀ of 0.3. For each strain, two cultures were adjusted to an OD₆₀₀ of 0.03 with NY; one was incubated with bacteriophagae 7-7-1 at an MOI of 1.0, and the second was incubated as a control in a shaking incubator at 220 rpm and 30°C. OD₆₀₀ readings were recorded after 24 h, and each experiment was run in quadruplicate.

**Transposon mutagenesis and selection of phage-resistant mutants.** Mobilization of plasmid pJG110 from *E. coli* DH5α to *Agrobacterium* sp. H13-3 RU12/001 was accomplished by triparental mating using *E. coli* DH5α harboring pBR600 as a helper strain essentially as described by Griffiths and Long (84). Each strain was scraped from freshly streaked TYC or LB agar plates and suspended as dense suspensions in 200 µL LB. One hundred microliters of a recipient strain and 5 µl (each) of helper and donor strains were mixed, and 100 µl of the mixed suspension was spread on an LB agar plate and incubated at 30°C for 20 h. The bacterial lawn was scraped off the plate, suspended in 3 ml LB containing 10% glycerol, and stored at −80°C. A 0.5-ml aliquot of the triparental-mating mixture was diluted in TYC containing streptomycin and neomycin to an OD₆₀₀ of 0.1. The culture was grown for 6 h at 30°C to an OD₆₀₀ of 0.2. The motility of cells was confirmed through light microscopic examination. Phage was added to an MOI of 1.0, and the culture was shaken for 16 h at 30°C. The cells were sedimented by centrifugation, suspended in 3 ml LB containing 10% glycerol, and stored at −80°C. Transposants were selected by plating serial dilutions on TYC agar plates containing streptomycin and neomycin and incubation for 3 days at 30°C. Individual colonies were spotted on Bromfield swim plates and incubated for 24 h at 30°C. Colonies that formed a swim ring were spotted on TYC agar plates containing streptomycin and neomycin. Motile colonies on Bromfield swim plates as a second screening.

**Chromosomal DNA preparation.** *Agrobacterium* sp. H13-3 DNA was isolated from 4-ml TYC cultures grown for 36 h at 30°C. The cells were sedimented by centrifugation; suspended in 500 µL 25 mM EDTA, pH 8.0, and mixed with 400 µL 20% (wt/vol) sucrose in 1 mM EDTA, 10 mM Tris-HCl, pH 8.0. After incubation for 30 min at −30°C, 10 µl 10-mg/ml RNase and 10 µl 100-mg/ml lysozyme were added to the thawed cell suspension and incubated for 45 min at 37°C. One hundred fifty microliters 5% sodium lauryl sarcosinate and 4 µl 100-mg/ml proteinase K were added, followed by overnight incubation at 50°C. After addition of 100 µl 3 M sodium acetate, pH 5.2, the mixture was extracted with an equal volume of phenol twice and with 24:1 chloroform-isoamyl alcohol once. Chromosomal DNA was precipitated by the addition of an equal volume of isopropanol and sedimented by centrifugation. The DNA pellet was washed with 70% ethanol and suspended in 100 µL H₂O.

**Genetic manipulations.** Transposon insertion sites were identified by arbitrary PCR (85) essentially as described by Griffiths and Long (84). Bacterial template DNA was used at a concentration of 0.2 ng/µl with oligonucleotides TSP1 (GGTTAATTTCCGCTTCCCCAAT) and ARB1A (GCCACGCGTCGACTAGTACN NNNNNNNNNNACCG) or ARB1B (GCCACCGCGTCGACTAGTACNNNNNNNNNTGGGG) in a first-round reaction with Taq polymerase (NEB) under the following conditions: 94°C for 3 min; six cycles of 94°C for 20 s, 33°C for 20 s, 70°C for 1 min; and 30 cycles of 94°C for 20 s, 43°C for 20 s, and 70°C for 1 min. In a second round, primers TSP2 (AGCTGCCCAATTCCGTTCCGTTG) and ARB2 (GCCACCGCGTCGACTAGTAC) were used to amplify from a 30-fold dilution of first-round reaction mixture under the following cycling conditions: 94°C for 3 min and 30 cycles of 94°C for 20 s, 52°C for 20 s, and 57°C for 1.5 min. The PCR products were sequenced using primer TSP2. Plasmid DNA was purified with a Wizard Plus SV Miniprep system (Promega), and PCR products were purified using the overlap extension PCR method described by Higuchi (86). These constructs were cloned into the mobilizable suicide vector pK18mobsacB, which was then used to transform *E. coli* S17-1, and conjugally transferred to *Agrobacterium* sp. H13-3 by filter mating according to the method of Griffitts and Long (84). Allelic replacement was achieved by sequential selections on neomycin and 10% sucrose, as described previously (88). Confirmation of allelic replacement and elimination of the vector was obtained by gene-specific primer PCR and DNA sequencing. The broad-host-range plasmid pBR1MCS-3 was utilized to complement transposon insertion strains. Broad-host-range plasmids were used to transform *E. coli* S17-1 and then transferred conjugally to *Agrobacterium* sp. H13-3 by streptomycin-tetracycline or streptomycin-neomycin double selection (89).

**Motility assays.** Swin plates containing Bromfield medium and 0.3% Bacto agar were inoculated with 3-µl droplets of the test culture and incubated at 30°C for 4 days. Motile-cell samples were observed with a Nikon Eclipse E600 phase-contrast microscope.

**Lipopolysaccharide purification and analysis.** Rough and smooth LPS were essentially purified according to the method of Johnson and Perry (90). Motile bacteria were harvested from a 1-liter TYC culture at an OD₆₀₀ of 0.3 via centrifugation at 10,000 × g for 15 min at 4°C. The cells were washed with 1 ml of phosphate-buffered saline (100 mM NaCl, 80 mM Na₂HPO₄, 20 mM NaH₂PO₄, pH 7), centrifuged as described above, and suspended in 25 ml of 50 mM Na₂PO₄, 5 mM EDTA, 0.05% (wt/vol) NaN₃. The suspensions were blended using a Waring blender at top speed for 1 to 2 min and stirred for 16 h at 4°C in the presence of 0.1 g of hen egg white lysozyme. The suspensions were further incubated for 20 min at 37°C and blended at top speed for 3 min. After the volume was adjusted to 100 ml using 20 mM MgCl₂, bovine pancreas RNase and DNase were added to reach a final concentration of 1 to 2 µg/ml, and samples were incubated for 10 min at 37°C, followed by a 10-min incubation at 60°C. After addition of proteinase K to a final concentration of 25 µg/ml, samples were incubated for 1 h at 60°C. The samples were then heated to 65 to 70°C, and an equal volume of 95% phenol was added. After 15 min of vigorous stirring, extracts were cooled to 15°C. Separation was achieved by centrifugation at 18,000 × g for 15 min
at 4°C. The top aqueous phase was removed and transferred to a new tube. An equal volume of water was added, and extraction with phenol was repeated. The aqueous phases were pooled and dialyzed against 4 liters of H2O until phenol odor was no longer recognized (approximately 3 or 4 changes). LPS was then lyophilized and suspended in H2O to reach a concentration of 5 mg/ml. To disperse aggregates and remove particulate matter, suspensions were heated to 65°C and centrifuged at 12,000 × g for 10 min at 4°C. Samples underwent ultracentrifugation at 105,000 × g for 3 h at 4°C. The centrifugation step was repeated until the content of protein and nucleic acid in supernatants was less than 1%. The supernatant was lyophilized and used as LPS stock. Twenty microliters Laemmli sample loading buffer containing 5% (vol/vol) β-mercaptoethanol was added, and samples were incubated for 5 min at 100°C. Separation and staining of LPS in polyacrylamide gels were performed as described previously (91, 92). After separating 2 μL LPS in a 15% SDS acrylamide gel at 20 mA, the gel was incubated in fixation solution (40% ethanol, 5% acetic acid). The fixation solution was replaced with oxidation solution (40% ethanol, 5% acetic acid, 0.7% periodic acid), and the gel was incubated on a rotary shaker at 40 rpm for 5 min. After washing three times with 1,000 ml distilled H2O at 50 rpm for 15 min, the gel was incubated at 70 rpm for 10 min in staining solution (19 mM NaOH, 197 mM NH4OH, 0.67% AgNO3) and washed three times with 1,000 ml distilled H2O at 40 rpm for 15 min. To develop the silver stain, the gel was incubated in developer solution (0.005% [wt/vol] citric acid, 0.05% [vol/vol] formaldehyde) at 45 rpm for 2 to 5 min and then washed three times with 1,000 ml distilled H2O for 15 min before documentation.

**Polysaccharide staining.** TYC plates were supplemented with either Congo red or calcofluor white stain to a final concentration of 150 μg/ml or 20 μg/ml, respectively. Overnight cultures were diluted with TYC medium to reach an OD600 of 0.3, and 5 μl of each was spotted onto each plate. The plates were then incubated for 4 days at 30°C and imaged.

**MALDI-TOF mass spectrometry.** Purified LPS samples were analyzed, essentially as described for whole-cell analyses (63), using 2,5-dihydroxybenzoic acid (DHB) as a matrix. Samples were prepared in sandwich format and analyzed with an ABIscie 4800 proteomics analyzer using the reflectron in negative-ion mode.

**Adsorption assays.** Overnight bacterial cultures in TYC were diluted with TYC to an OD600 of 0.3 and a final volume of 10 ml. The cells were evaluated for motility through light microscopic examination. Phage was added at an MOI of 0.01, and the cultures were incubated for 10 min at 30°C to allow adsorption to occur. As a control, TYC medium was mock treated with the same amount of phage as described above. A 1-ml aliquot of infected cultures and the control was centrifuged at 15,000 × g for 3 min at 4°C. Serial dilutions of each supernatant were mixed with 100 μl of Agrobacterium sp. H13-3, incubated for 5 min at room temperature, and mixed with 4 ml of molten TYC containing 0.5% agar. This mixture was layered onto TYC plates and incubated overnight at 30°C to enable plaque formation. The adsorption percentages were calculated using the following formula: \(1 - \frac{\text{phage titer of supernatant after cells removed/ phage titer of control reaction mixture without bacterial cells}}{100}\).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00363-18.

**SUPPLEMENTAL FILE 1,** PDF file, 0.7 MB.

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