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Host Exopolysaccharide Quantity and Composition Impact *Erwinia amylovora* Bacteriophage Pathogenesis

Dwayne R. Roach,[a,b]* David R. Sjaarda,[a,b] Alan J. Castle,[a] Antonet M. Svircev[b]

Department of Biological Science, Brock University, St. Catharines, Ontario, Canada[a]; Agriculture and Agri-Food Canada, Vineland Station, Ontario, Canada[b]

Erwinia amylovora **bacteriophages (phages) belonging to the *Myoviridae* and *Podoviridae* families demonstrated a preference for either high-exopolysaccharide-producing (HEP) or low-exopolysaccharide-producing (LEP) bacterial hosts when grown on artificial medium without or with sugar supplementation. *Myoviridae* phages produced clear plaques on LEP hosts and turbid plaques on HEP hosts. The reverse preference was demonstrated by most *Podoviridae* phages, where clear plaques were seen on HEP hosts. Efficiency of plating (EOP) was determined by comparing phage growth on the original isolation host to the that on the LEP or HEP host. Nine of 10 *Myoviridae* phages showed highest EOPs on LEP hosts, and 8 of 11 *Podoviridae* phages had highest EOPs on HEP hosts. Increasing the production of EPS on sugar-supplemented medium or decreasing production by knocking out the synthesis of amylovoran or levan, the two EPSs produced by *E. amylovora*, indicated that these components play crucial roles in phage infection. Amylovoran was virtually essential for proliferation of most *Podoviridae* phages when phage population growth was compared to the wild type. Decreased levan production resulted in a significant reduction of progeny from phages in the *Myoviridae* family. Thus, *Podoviridae* phages are adapted to hosts that produce high levels of exopolysaccharides and are dependent on host-produced amylovoran for pathogenesis. *Myoviridae* phages are adapted to hosts that produce lower levels of exopolysaccharides and host-produced levan.**

The common characteristic of infection of rosaceous plants by *Erwinia amylovora*, the fire blight pathogen, is the appearance of wilt, necrosis, and the production of copious amount of exudates, or "ooze." In the 1930s, experiments established that the ooze was bacterial in origin and responsible for the induction of wilt symptoms in pear shoot cuttings (1). A majority of the field samples of *E. amylovora* examined by Billing (2) were encapsulated by the ooze, with less than 1% having no capsule. Goodman et al. (3) named the ooze polysaccharide amylovorin, to reflect its toxic effect on plant tissues. Amylovorin was subsequently renamed amylovoran to be consistent with polysaccharide nomenclature (4). In due course, the linkages between *E. amylovora*'s capsule, slime, polysaccharides, ooze, and pathogenicity were made (5, 6, 7, 8, 9, 10). Growth of the bacterium on sucrose-, glucose-, or sorbitol-enriched media results in the production of excess quantities of two exopolysaccharides (EPSs), one acidic and one neutral (2, 6, 7). The acidic EPS, amylovoran, may form a capsule, slime, and/or float free in the liquid medium (5, 11). Amylovoran is a heterogeneous polymer consisting of repeating units of one glucuronic acid and four galactose residues (12). The *ams* region of the genome controls the production of the EPS (13), with *rcsA* and *rcsB* genes being required for synthesis (13, 14). Mutation in *rcsA* or *rcsB* results in reduced amylovoran production (15). The neutral EPS, levan, is synthesized in the presence of sucrose by the enzyme levansucrase, which cleaves the sugar and polymerizes fructose into a polyfructan (β-2,6-D-fructofuranan) (10, 11, 16). Levan synthesis takes place extracellularly through the action of the enzyme encoded by the *lsc* gene (10, 17).

EPSs play multifaceted and complex roles in the interactions between bacteria and their environments (18, 19). Functions include key roles in bacterial virulence and pathogenesis (20, 21, 22, 23, 24), surface adhesion (18), as a major constituent of the biofilm glycocalyx (18), and as a component that renders the cell susceptible (25) or resistant (26, 27, 28) to bacteriophage (phage) attack. EPSs prevent cellular desiccation (3, 25, 29, 30) by keeping nutrients and water in close proximity to the bacterial cell (29, 30). The protective nature of the EPSs of *E. amylovora* is exhibited in the ability of the pathogen to "hide" from the plant host defenses (22). Amylovoran mutants are avirulent, producing no disease symptoms (4, 11), and thus EPS is an essential contributor to *E. amylovora* pathogenicity (4, 23) and virulence (2, 6, 7, 15, 20, 21). Mutants deficient for levansucrase produce disease symptoms on immature pears and therefore remain pathogenic (10, 17). EPSs also play an important role in bacterial biofilms, and Koczan et al. (19) showed that, for *E. amylovora*, both amylovoran and levan are required for biofilm formation. *In vitro*, cells lacking amylovoran were unable to attach to a growth substrate to initiate biofilm formation, and levan-deficient mutants showed reduced biofilm formation.

EPSs have been reported to delay or prevent phage adsorption for several bacterial species, presumably by providing a physical barrier between the phage and outer membrane receptors (26, 27, 28). Certain phages, however, have evolved to recognize specifically and bind bacterial extracellular polymers (31, 32, 33, 34). *Erwinia* spp. phages normally produce clear translucent plaques indicative of a strong infection *in vitro* (6, 7, 20, 35, 36, 37, 38). Many phage infections, however, result in the production of turbid or hazy plaques (2, 36, 37, 38) that contain a thin layer of

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Address correspondence to Alan J. Castle, acastle@brocku.ca.
* Present address: Dwayne R. Roach, Animal Biosciences and Biotechnology Lab, ARS, U.S. Department of Agriculture, Beltsville, Maryland, USA.
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bacteria on the plaque surface. Historically, hazy plaques were attributed to the presence of lysogenic bacteria resulting from infections with temperate phages (2, 37, 38). The main purpose of this study was to determine the roles of amylovoran and levan in plaque morphology, preference of phages for specific hosts, and phage productivity. Lytic phages are currently being evaluated as biological control agents for the control of *E. amylovora* in the orchard (35, 36, 39, 40, 41, 42). The flower stigma is the primary entry site for *E. amylovora* during fire blight infection and provides the nutrient-rich environment in which the pathogen and biological control agent interact (43, 44, 45, 46). This sugar-rich microenvironment stimulates EPS production by the bacterium and could potentially alter the efficacy of a phage-based biopesticide. The present work provides information that is critical to the selection of phages developed as biocontrol mixtures or cocktails. The choice of phages will directly influence field efficacy and the prevention of bacterial resistance.

MATERIALS AND METHODS

Bacterial isolates, bacteriophages, and plasmids. Bacterial isolates, phages, and plasmids used in this study are listed in Table 1. Phage isolates were stored in 0.8% nutrient broth (Difco) at 4°C. The phages, part of the AAFC Vineland Master Collection, were characterized previously by restriction fragment length polymorphism analysis and transmission electron microscope observations into groups and families (36). New additions were placed into families based on information from real-time PCR probe-primer sets designed to detect *Erwinia* spp. phages belonging to the *Myoviridae* and *Podoviridae* (39, 47). *E. amylovora* was grown in nutrient broth (NB; Difco Laboratories, Sparks, MD), nutrient agar (NA; Laboratories, Sparks, MD), NA supplemented with 0.5% sucrose (NAS), or Luria-Bertani medium (LB; Difco Laboratories, Sparks, MD) at 27°C. To increase EPS production, NB was supplemented with 0.5% sucrose and 1% sorbitol (NBSS). For transformation selection, antibiotics were added to the culture medium at the following concentrations: kanamycin (Km), 20 μg/ml; chloramphenicol (Cm), 20 μg/ml; ampicillin (Ap), 100 μg/ml. Datsenko and Wanner (48) previously detailed the properties of the plasmids used in the present study for the production of deletion mutants.

Phage manipulations. The host ranges of *Erwinia* spp. phages were determined by the ability to form plaques on *E. amylovora* isolates and by plaque morphology. Clear plaques indicated high host sensitivity, turbid plaques indicated partial lysis, and no plaques indicated a nonhost. Efficiency of plating (EOP), the ratio of PFU/ml obtained with an assay host to the PFU/ml obtained with the isolation host, was calculated using the double layer plaque method (49). Assay host refers to the tested host, and isolation host refers to the *E. amylovora* isolate with which Gill et al. (36) initially isolated the phage. Phage population growth was monitored by inoculating liquid medium with phage and bacteria at a multiplicity of infection of 1. The cultures were incubated for 24 h at 27°C, lysates were prepared, and titers were determined by using the double agar overlay method (49).

Generation of EPS-deficient mutants. A modified PCR deletion method (24, 48) was used to generate *E. amylovora* mutants with deletions of the *rscB* gene (Δ*rscB*), the regulator of capsular synthesis, the *lsc* gene (Δ*lsc*), which encodes levansucrase, or double deletions of both genes (Δ*rscB* Δ*lsc*). For mutant production, linear recombination constructs consisting of either a kanamycin (Km^r) or chloramphenicol (Cm^r) resistance gene flanked by 50-nucleotide homology arms targeting either *lsc* or *rcsB* were created by PCR. The Km^r gene was amplified from pKD13 with the *rcsB*-Km primers, and the Cm^r gene was amplified from pKD32 (48) with the *lsc*-Cm primers. Plasmids (obtained from The *E. coli* Genetics Stock Center, Yale University) and primers used to generate recombination constructs are listed in Table 1 and Table S1 in the supplemental material, respectively. Each PCR mixture contained final concentrations of 200 nM each primer, 1× polymerase buffer (NEB, Ipswich, MA), 2.5 U *Taq* polymerase (NEB), 300 μM each deoxynucleoside triphosphate (NEB), 3 mM $MgCl_2$, and 2 μl of DNA template. Reactions were run in a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA) under the following conditions: 95°C for 5 min, and 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s. PCR products were purified using a gel purification kit (Norgen Biotek, St. Catharines, ON, Canada).

Prior to introduction of a recombination construct, *E. amylovora* cells were made electrocompetent by washing 3 times in 35 ml ice-cold sterile distilled H_2O and transformed with plasmid pKD46 by electroporation. Resulting transformants were screened by selecting for ampicillin resistance on LB agar. The pKD46 transformants were grown overnight at 27°C, reinoculated in LB broth containing 0.1% arabinose to induce the *red* recombinase (48), grown to exponential phase (optical density at 600 nm [OD_{600}], 0.8), and made electrocompetent again by washing as described above. Cells were transformed with a recombination construct using electroporation and recovered in 1 ml of SOC medium (50) for 1 to 4 h at 27°C before plating on LB agar with the appropriate selective antibiotic. Electroporations were performed at 2.5 kV and 25 μF with the pulse controller set at 200 Ω. Recombinants were selected on LB containing kanamycin or chloramphenicol. In the resulting mutants (Table 1), the Cm^r or Km^r insert replaced part of the coding region of the target gene

TABLE 1 *Erwinia amylovora* isolates, bacteriophages, and plasmids used in the study

Isolate or plasmid	Description[a]	Reference(s) or source
Erwinia amylovora		
Ea110R[b]	Wild type	37
Ea110RΔ*rcsB*	Km^r, Δ*rcsB*	47
Ea110RΔ*lsc*	Cm^r, Δ*lsc*	47
Ea29-7	Wild type	36
Ea29-7Δ*rcsB*	Km^r, Δ*rcsB*	47
Ea29-7Δ*lsc*	Cm^r, Δ*lsc*	47
EaD-7	Wild type	51
EaD-7Δ*rcsB*	Km^r, Δ*rcsB*	This study
EaD-7Δ*lsc*	Cm^r, Δ*lsc*	This study
Ea6-4	Wild type	51
Ea6-4Δ*rcsB*	Km^r, Δ*rcsB*	47
Ea6-4Δ*lsc*	Cm^r, Δ*lsc*	47
Ea6-4Δ*rcsB* Δ*lsc*	Km^r Cm^r, Δ*rcsB* Δ*lsc*	47
Ea17-1-1	Wild type	51
Ea17-1-1Δ*rcsB*	Km^r, Δ*rcsB*	47
Ea17-1-1Δ*lsc*	Cm^r, Δ*lsc*	This study
Ea17-1-1Δ*rcsB* Δ*lsc*	Km^r Cm^r, Δ*rcsB*, Δ*lsc*	This study
EaG-5	Wild type	51
Ea G-5Δ*rcsB*	Km^r, Δ*rcsB*	This study
EaG-5Δ*lsc*	Cm^r, Δ*lsc*	47
Ea G-5Δ*rcsB* Δ*lsc*	Km^r Cm^r, Δ*rcs*, Δ*lsc*	This study
Bacteriophages		
ϕEa21-4	*Myoviridae*	40
ϕEa1(h)	*Podoviridae*	37
ϕEa35-7	*Siphoviridae*	36
46 phage isolates	All three families	36, 47
Plasmids		
pKD46	Ap^r, P_{BAD} γ β exo pSC101 oriTS	48
pKD13	Km^r, FRT cat FRT PS1 PS2 oriR6K rgbN	48
pKD32	Cm^r, FRT cat FRT PS1 PS2 oriR6K rgbN	48

[a] Δ*rcsB*, amylovoran deficient; Δ*lsc*, levan deficient.
[b] Rifampin-resistant isolate of *E. amylovora* 110.

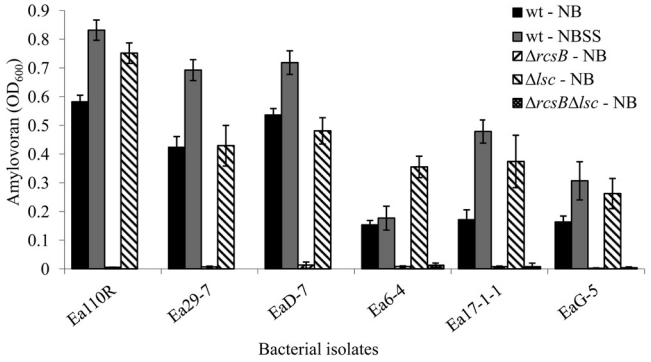

FIG 1 Production of amylovoran by the wild type and Δ*rscB*, Δ*lsc*, and Δ*rscB* Δ*lsc* mutants of *Erwinia amylovora*. Amylovoran was precipitated from culture filtrates with 50 mg/ml of cetylpyridinium chloride, and turbidity (the OD_{600}) was measured (5). Each bar represents the mean ± standard deviation of three independent measurements.

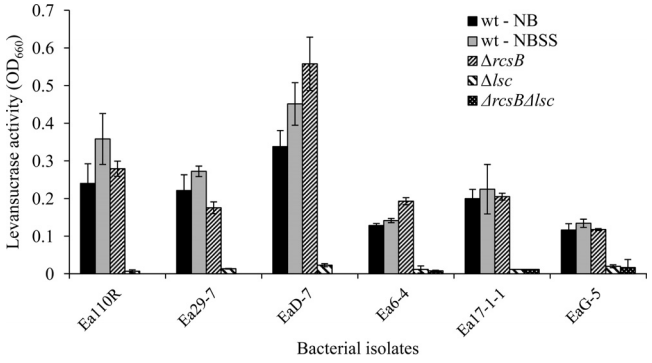

FIG 2 Production of levansucrase by the wild type and Δ*rscB*, Δ*lsc*, and Δ*rscB* Δ*lsc* mutants of *Erwinia amylovora*. Activities were measured in the culture medium after removal of the bacterial cells by centrifugation (15). Each bar represents the mean ± standard deviation of three independent measurements.

by site-specific recombination in the flanking regions. To evict the temperature-sensitive pKD46 from mutants, cells were briefly heated to 37°C for 10 min, plated on LB lacking ampicillin, and grown overnight at 27°C. Replica plating on LB and LB-ampicillin agar was used to differentiate the colonies that lost the ampicillin resistance carried on pKD46.

EPS measurement. Levan and amylovoran production levels were measured in 1-ml supernatant aliquots obtained from liquid cultures grown in nutrient broth (Difco) at 27°C with shaking. Levan production was not measured directly. Instead, levansucrase in supernatants was detected by the addition of 1 ml of 50 mM sodium phosphate, 2 M sucrose, and 0.05% sodium azide and incubation for 24 h at 27°C (15). The amylovoran concentration was determined by precipitating the substance from the culture filtrates with 50 mg/ml of cetylpyridinium chloride and measuring turbidity (the OD_{600}) (5).

RESULTS

EPS production in wild-type isolates. Wild-type *E. amylovora* isolates Ea110R, Ea29-7, and EaD-7 produced higher levels of amylovoran in NB than isolates Ea6-4, Ea17-1-1, and EaG-5 (Fig. 1). Amendment of the growth medium with 0.5% sucrose and 1% sorbitol resulted in increased amylovoran in all isolates except for Ea6-4. Levan production, measured by levansucrase activity, was higher for Ea110R, Ea29-7, and EaD-7 and lower for Ea6-4, Ea17-1-1, and EaG-5 (Fig. 2). Addition of sorbitol and sucrose to the medium resulted in no statistical difference for Ea110R, Ea29-7, and EaD-7 and modest increases in levansucrase activity for Ea6-4, Ea17-1-1, and EaG-5. Based on these results, Ea110R, Ea29-7, and EaD-7 were grouped as high EPS producers (HEP), and Ea6-4, Ea17-1-1, and EaG-5 were considered low EPS producers (LEP).

EPS production and plaque morphology. *Myoviridae* phages, classified as group 1 (36), produced clear plaques on the LEP hosts (Ea6-4, Ea17-1-1, and EaG-5) (Table 2). On HEP hosts (Ea110R, Ea29-7, and EaD-7), these phages either produced turbid plaques or completely failed to infect. The opposite pattern was found for most (8 of 11) of the *Podoviridae* phages. These phages produced large clear plaques on HEP isolates and turbid or no plaques on LEP hosts. The analysis included only two *Siphoviridae* phages, and no consistent pattern for host preference was evident, as ϕEa10-5 produced clear plaques on all hosts and ϕEa35-7 produced clear plaques only on HEP hosts. To determine if plaque morphology would be changed by host modification via passing the phage through a nonpreferred host, four phages were grown in Ea110R (HEP group) and Ea6-4 (LEP group). Plaque appearance was unchanged on the same six hosts listed in Table 2 for any phage grown in either host. Thus, plaque morphologies on either host type were not altered by the passage through a nonpreferred host for any of the four phages (data not shown).

Plaque morphology and efficiency of plating. To correlate plaque morphology with sensitivity or partial resistance to a phage, EOP was determined for several phage-host combinations (Table 2; EOP values are indicated in parentheses after the plaque morphology descriptor). The average EOP ($n = 24$; combinations where the plaques were too faint to be counted accurately were excluded from this total) of *Myoviridae* phages on LEP hosts Ea6-4, Ea17-1-1, and EaG-5 was 1.08, and on HEP hosts Ea110R, Ea29-7, and Ea D-7 the average EOP ($n = 23$) was 0.48. This difference was significant ($P < 0.001$; Mann-Whitney U test). *Podoviridae* phages had an average EOP of 0.85 on LEP hosts ($n = 13$) and 1.06 on HEP hosts ($n = 33$), a significant difference ($P < 0.05$). For *Siphoviridae*, ϕEa35-7 had a preference for high-EPS hosts (EOPs of 0.9 for HEP and 0 for LEP), and for ϕEa10-5 the EOP was 2.0 on the LEP hosts and 0.7 on HEP isolates. A strong correlation between plaque morphology and EOP was evident. Of the 71 phage-host combinations that resulted in clear, countable plaques, 60 (85%) had EOP values greater than 0.7. Only 8 of the 23 combinations (35%) that produced turbid yet countable plaques reached this criterion.

Enhanced EPS production and phage population growth. Single representatives from the three phage families were monitored for population propagation on five *E. amylovora* hosts on NA or NAS to increase EPS production (Fig. 3). The *Myoviridae* phage ϕEa21-4 multiplied equally well on Ea29-7 (HEP) and Ea6-4, Ea17-1-1, and EaG-5 (all LEP), but substantially less well with the HEP isolate Ea110R (Fig. 3A). With the exception of the growth on Ea110R, these outcomes were consistent with EOP observations, which indicated that ϕEa21-4 was able to infect most hosts equally well. Growth on NAS increased titers 2.3-fold (NA versus NAS, $P = 0.02$), a result that did not correlate well with plaque morphology, as this phage produced clear plaques on LEP hosts. This increase, however, was far smaller than the responses of the other two phages (see below) and may simply have been due to increased vigor of the hosts on the more-nutritive medium. The

TABLE 2 Effects of *E. amylovora* HEP and LEP cells on bacteriophage plaque appearance and efficiency of plating

Family and phage isolate	Isolation host[a]	Phage group[b]	Plaque appearance (EOP)[c]						Clear plaque formations[d]:
			HEP			LEP			
			Ea110R	Ea29-7	EaD-7	Ea6-4	Ea17-1-1	EaG-5	
Myoviridae									
ϕEa9-6	Ea17-1-1	1*	T (0.5)	N (0)	T (0.1)	C (1.0)	C (1.0)	C (1.4)	LEP
ϕEa9-7	Ea6-4	1*	T (1.0)	T (1.0)	T (0.2)	C (1.0)	C (1.4)	C (1.0)	LEP
ϕEa10-1	Ea17-1-1	1*	T (0.3)	T (—)	T (0.1)	C (0.8)	C (1.0)	C (0.5)	LEP
ϕEa10-2	Ea6-4	1	T (0.5)	N (0)	T (0.4)	C (1.0)	C (1.9)	C (1.0)	LEP
ϕEa10-4	EaG-5	1	T (0.4)	T (0.6)	T (0.3)	C (0.6)	C (1.8)	C (1.0)	LEP
ϕEa21-2	EaG-5	1	T (1.0)	T (0.3)	T (0.8)	C (1.3)	C (1.5)	C (1.0)	LEP
ϕEa21-4	Ea6-4	1	T (1.2)	T (1.3)	T (0.7)	C (1.0)	C (1.2)	C (0.9)	LEP
ϕEa35-2	Ea17-1-1	1	T (0.3)	N (0)	N (0)	C (0.6)	C (1.0)	C (0.9)	LEP
Siphoviridae									
ϕEa10-5	Ea110R	[2]	C (1.0)	C (0.5)	C (0.6)	C (1.6)	C (1.9)	C (2.5)	Both
ϕEa35-7	Ea29-7	2	C (0.8)	C (1.0)	C (0.9)	N (0)	T (—)	T (—)	LEP
Podoviridae									
ϕEa10-8	Ea29-7	3A	C (1.5)	C (1.0)	C (1.0)	T (—)	T (—)	T (—)	HEP
ϕEa10-11	Ea17-1-1	3A	C (0.5)	C (0.2)	C (0.3)	C (0.5)	C (1.0)	C (0.6)	Both
ϕEa31-3	Ea29-7	3A	C (1.0)	C (0.9)	C (0.7)	N (0)	T (0.3)	N (0)	HEP
ϕEa46-2	EaD-7	3B	C (1.3)	C (1.1)	C (1.0)	T (—)	T (—)	T (—)	HEP
ϕEa1(h)[e]	Ea110R	3C	C (1.0)	C (2.3)	C (1.2)	C (2.6)	T (—)	C (3.2)	Both
ϕEa9-4	EaG-5	5	T (0.5)	N (0)	T (0.2)	C (0.3)	C (0.9)	C (1.0)	LEP
ϕEa51-4	Ea29-7	6	C (1.5)	C (1.0)	C (0.9)	T (—)	T (—)	T (—)	HEP
ϕEa31-7	Ea29-7	*	C (0.9)	C (1.0)	C (1.4)	T (—)	T (—)	T (—)	HEP
ϕEa31-8	EaD-7	*	C (1.5)	C (0.9)	C (1.0)	T (—)	T (—)	T (—)	HEP
ϕEa45-1B	Ea29-7	*	C (0.8)	C (1.0)	C (1.0)	N (0)	T (—)	T (—)	HEP
ϕEa46-1A2	EaD-7	*	C (3.0)	C (2.5)	C (1.0)	T (—)	T (0.7)	T (—)	HEP

[a] Original bacterial isolation host, as published in reference 36.
[b] Bacteriophages were placed into families and groups based on RFLP patterns and transmission electron micrographs (36). * denotes the sole use of real-time PCR to place the phage into the *Myoviridae* or *Podoviridae* (47); [2] indicates a presumptive *Siphoviridae* phage based on nondetection by real-time PCR (47).
[c] Plaque appearance categories: C, clear; T, turbid/hazy; N, no plaques. EOP is the phage titer on the bacterial isolate divided by the phage titer on the isolation host.
[d] General category of bacterial host, based on HEP or LEP status. —, plaques were present but could not be counted and distinguished due to their hazy appearance.
[e] Ea1(h) was obtained from A. L. Jones (38).

Siphoviridae phage ϕEa35-7 (Fig. 3B) propagated best on the HEP hosts Ea110R and Ea29-7 when grown on NA (HEP versus LEP, $P < 0.001$), a finding consistent with clear plaque morphology and high EOP results (Table 2). Phage production increased dramatically in four of the five cultures grown on supplemented medium. The average population size for all five hosts was approximately 30 times higher on NAS than on nonsupplemented NA ($P < 0.001$), a result consistent with the observation that this phage preferred hosts that produce large amounts of EPS. The contribution of improved host vigor on NAS to increased phage production cannot be determined presently. However, based on the magnitude of the change compared to ϕEa21-4, it is most probable that the major factor in the increase was enhanced EPS production. The *Podoviridae* phage ϕEa1(h) (Fig. 3C) produced substantially fewer progeny, compared to the other two phages, on all hosts, but it showed significantly more production on HEP hosts in the absence of sucrose (HEP versus LEP, $P < 0.01$) or presence of sucrose (HEP versus LEP, $P < 0.001$). The addition of sucrose to the growth medium had a strong effect and increased phage titers on all hosts, with an average increase of almost 6-fold (NA versus NAS, $P < 0.01$). As with the *Siphoviridae* phage, the contribution of increased host vigor cannot be estimated accurately, but it is likely that most of the increase is a result of increased EPS. The similar production on all hosts was consistent with clear plaque formation and strong EOPs for most isolates (Table 2).

EPS-deficient mutants, decreased EPS production, and phage population growth. Mutants with defects in the synthesis of amylovoran and/or levan were produced in order to understand the roles of each of the two EPSs in phage infection. Amylovoran-deficient mutants with a deletion of the *rcsB* gene and levan-deficient mutants with a deletion of the *lcs* gene were generated for all isolates (Table 1). Stable *rcsB* and *lsc* double deletion derivatives were recovered from the LEP isolates Ea6-4, Ea17-1-1, and EaG-5. Despite numerous attempts, including several alterations to the mutagenesis procedures, double deletion mutants of the HEP isolates were not obtained, suggesting that this genotype is lethal in these bacteria. All mutational changes were confirmed by PCR.

Amylovoran production was negligible in all Δ*rcsB* strains (Fig. 1), and levansucrase activity was almost undetectable for all Δ*lsc* strains (Fig. 2). The Δ*rcsB* Δ*lsc* double mutants produced extremely low amounts of both EPSs. Four of the six levansucrase mutants, Ea110RΔ*lsc*, Ea6-4Δ*lsc*, Ea17-1-1Δ*lsc*, and EaG-5Δ*lsc*, demonstrated increases in amylovoran accumulation compared to their wild-type progenitor. Most *rcsB* mutants did not show any significant change in levansucrase activity compared to the nonmutant wild type.

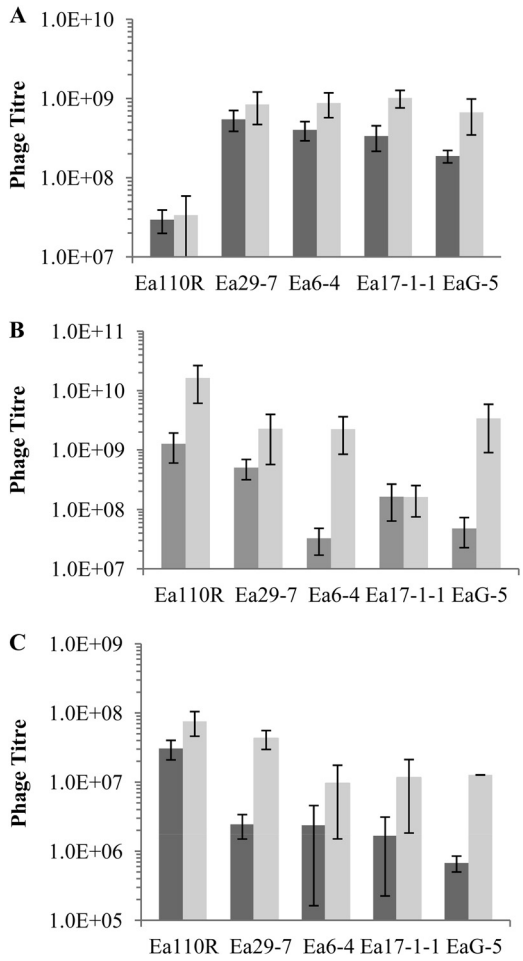

FIG 3 Population titers of *Erwinia amylovora* bacteriophages from five host isolates grown on NA or NAS. (A) ϕEa21-4 (*Myoviridae*); (B) ϕEa35-7 (*Siphoviridae*); (C) ϕEa1(h) (*Podoviridae*). Results are means and standard deviations from three independent experiments.

Phage populations were substantially decreased for all phages on either Δ*rcsB* or Δ*lsc* strains (Table 3). *Myoviridae* and *Siphoviridae* phages produced significantly fewer progeny on the Δ*lsc* strains than on the Δ*rcsB* strains ($P < 0.001$ and $P < 0.01$, respectively). Decreased amylovoran production in the Δ*rcsB* strains resulted in significantly reduced progeny for all of the *Podoviridae* phages ($P < 0.001$). A few phages in all three families showed apparently reduced progeny production in both mutant backgrounds, although the results were not statistically significant.

DISCUSSION

The production of clear or turbid plaques on a host lawn appeared to be a good indication of the ability of a phage to attack an *E. amylovora* wild-type isolate. This characteristic correlated well with a high EOP and population growth for most phages. A possible explanation for the production of turbid plaques is that a portion of the population has undergone lysogeny, resulting in resistance to homologous phages (52, 53). However, none of the hosts contained detectable prophage DNA, based on real-time PCR screening and/or production of virions through induction (47). A more reasonable explanation for differences in plaque appearance is host preference. Billing (2) observed in 1960 that some phages produce confluent lysis and plaque halos on encapsulated *E. amylovora* isolates and turbid lysis on hosts with reduced encapsulation. Other phages showed the opposite pattern. In the present study, we found that most *Podoviridae* phages appear to be adapted to hosts that produce relatively high amounts of EPS and *Myoviridae* phages appear to be adapted to hosts that have lower levels of EPS (Table 2). Individual phages in the *Siphoviridae* may be adapted to one type of host or the other; however, no generalization on host preference can be made for this family due to the small sample size as well as the lack of a clear pattern within our sample.

Alteration of the amount of EPS produced, either by increasing production on supplemented medium or by knocking out synthesis of either type in the deletion mutants, indicated that phage efficacy is dependent on both of these substances. Amylovoran was virtually essential for the proliferation of most phages in the *Podoviridae* with progeny production dropping to less than 1% in the Δ*rcsB* mutants compared to wild-type progenitors. For *Myoviridae* and *Siphoviridae* phages, however, levan may be the primary site of interaction, as growth on the amylovoran knockout strain showed little to no decrease in progeny production while the levan knockout reduced proliferation significantly (Table 3). The individual roles of amylovoran and levan in *E. amylovora* phage-host interactions have not been described, and the involvement of EPS in *Erwinia* spp. phage infection is limited to a few early reports (2, 6, 7, 20). Some phages have evolved to recognize specific polysaccharides of other bacterial species (34, 54, 55). Certain *Escherichia coli* phages bind only when a capsular EPS with a serotype-specific surface K antigen is present on a potential host (54, 55, 56, 57). Removing the capsular EPS from cells results in a lack of K antigen-specific phage lysis (55). Phages that attack *Vibrio cholerae* have also been found to specifically lyse EPS-pro-

TABLE 3 Efficiencies of plating of bacteriophages from the *Myoviridae*, *Siphoviridae*, and *Podoviridae* on Δ*rcsB*, Δ*lsc*, and wild-type isolates of *Erwinia amylovora*

Family and phage isolate	Isolation host	Efficiency of plating[a]	
		Δ*rcsB*[b]	Δ*lsc*[c]
Myoviridae			
ϕEa10-1	Ea17-1-1	0.25	0.06
ϕEa21-2	Ea17-1-1	1.11	0.12
ϕEa21-4	Ea 6-4	1.38	0.02
Siphoviridae			
ϕEa10-19	Ea17-1-1	0.64	0.05
ϕEa35-7	Ea110R	5.34	0.26
Podoviridae			
ϕEa10-6	Ea17-1-1	0	0.98
ϕEa31-3	Ea29-7	0	0.34
ϕEa35-3	Ea110R	0.0005	0.23
ϕEa45-1B	Ea29-7	0.0007	1.84
ϕEa46-1A2	Ea110R	0.0003	0.34
ϕEa50-3	Ea29-7	0.0012	1.35
ϕEa51-7	Ea29-7	0.0026	1.44

[a] Number of plaques on the mutant host strain divided by the number of plaques on the wild-type bacterial host. Results are from three independent experiments.
[b] Amylovoran-deficient mutant.
[c] Levan-deficient mutant.

ducing strains (58), and the capsular polysaccharide surrounding the cells of *Streptococcus thermophilus* could also play a role in the cellular adsorption of specific phages (32). For *Erwinia* spp. phages, it is likely that amylovoran and levan serve as binding sites, similar to these examples.

Many EPS-specific phages carry depolymerases that can recognize and degrade specific polymers as part of the virion tail (56). *Podoviridae* phages carry depolymerases that degrade amylovoran (59, 60). Depolymerases are thought to allow the virion to gain access to the cell surface, where it most likely binds to an outer membrane receptor. Removal of the EPS barrier should improve phage growth by exposing an outer membrane receptor, but that was not found to be the case in the present study for phages that are known to produce depolymerase. If such a secondary receptor exists in *E. amylovora*, binding to EPS is a necessary step prior to attachment to the membrane receptor. There is the possibility that the *rcsB* deletion also altered some other component that acts as the cell surface receptor. There is evidence for similar secondary effects. Mutations in the *rcs* system of *E. amylovora* have been found to increase resistance to the antimicrobial peptide polymyxin B, which requires cell membrane binding to be effective (61).

Myoviridae and *Siphoviridae* phages do not express depolymerase activities in culture and appear to lack the gene (39, 40). It is not known how these phages subsequently bypass the EPS barrier and infect their hosts. Adaptation of these phages to LEP hosts appears to be a reasonable strategy for propagation, simply because the EPS barrier is smaller than that on an HEP bacterium.

For several bacterial species, most of the functions ascribed to EPS are of a protective nature, including inhibition of phage attack (28). EPS is presumed to provide a physical barrier between infecting phages and cell surface receptors (27, 62). A protective function has been reported for *Rhizobium meliloti*, as EPS nonspecifically prevented phage adsorption (8). The physical removal of the K1 EPS capsule restored phage susceptibility of *Escherichia coli* (57). EPS was also found to be responsible for the inhibition of phage adsorption to *Lactococcus lactis* (27). These observations suggest that the production of EPS may be a near-universal antiphage defense mechanism, providing a physical barrier to the cell surface receptors. The EPSs of *E. amylovora*, however, do not appear to provide such a protective role and may simply provide an attachment site.

Current information suggests that amylovoran and levan production levels are loosely coregulated. Amylovoran biosynthesis is controlled by the *ams* operon, which includes the *rcsA* and *rcsB* genes (61). Overexpression of *rcsA* or *rcsB* reduces levansucrase synthesis (10). Mutations that impair *rcsA* function can have variable effects on levan synthesis, reducing levansucrase activity or not. *rcsB* mutations generally do not effect levan production (15). The present results with the *rcsB* deletion strains that showed no change in levansucrase activities support this conclusion (Fig. 2). Mutation of the levansucrase gene, *lsc*, has been reported to have no effect on amylovoran production (10). Four of the *lsc* deletion mutants in this study, however, showed increases in amylovoran production, ranging from 29 to 119% (Fig. 1). The difference between the two studies can be explained by either an environmental effect, as Geier and Geider (10) tested production on medium supplemented with sorbitol to stimulate EPS production while the present measurements were on bacteria grown on nonsupplemented nutrient agar, or by unknown genetic differences in the isolates used in the two studies. While it is presently not possible to determine the nature of the cross talk between the two EPS biosynthetic pathways, it is apparent that isolates differ in overall EPS production but still strive to maintain a minimal level of at least one of the two types (15). The difficulty in recovering Δ*rcsB* Δ*lsc* strains of the HEP isolates supported this conclusion. Furthermore, coregulated synthesis of the EPSs would allow the bacteria to avoid unnecessary energy expenditures associated with EPS overproduction.

This study showed that the pathogenicity and presumably adsorption of phages that attack *E. amylovora* was mediated by amylovoran or levan. If in fact the phage receptor for certain *Erwinia* spp. phages is amylovoran, the obvious way for the cell to become phage resistant is to change the polymer's composition or abolish its production. However, amylovoran produced by *E. amylovora* is correlated with pathogenicity, as deficient mutants are avirulent (4, 5, 9, 10, 17, 21, 63, 64, 65). If amylovoran composition were altered by mutation, it would likely also reduce virulence of the bacterium to the plant host. This in turn would greatly reduce the chance of survival of the bacterium. Thus, the adaptation of phages in the *Podoviridae* to amylovoran is a very effective strategy for their own survival.

The fire blight pathogen populations increase exponentially in the sugar-rich stigma surface under optimal environmental conditions. The bacterial capsule, specifically the constituent EPS amylovoran, plays an important role in bacterial virulence and pathogenicity. Our research has demonstrated that *Erwinia* spp. phages are highly adapted to the pathogen and the plant environment. Phage populations increase significantly in the presence of sugars, and amylovoran plays a key role in the ability of the *Podoviridae* phages to infect the HEP bacterial host. In contrast, the *Myoviridae* phages preferentially infect the LEPs and respond to the presence of levan on the host. To obtain a phage-mediated biopesticide with high efficacy, these phage-host interactions will need to be taken into consideration.

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