APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1987, p. 677–682
0099-2240/87/040677-06$02.00/0

Vol. 53, No. 4

Effect of Increased β-Glucosidase Activity on Virulence of *Erwinia amylovora*

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Received 22 September 1986/Accepted 22 December 1986

Plant tissues often contain β-glucosides that can be enzymatically hydrolyzed to produce toxic aglycones. It has been suggested that the low β-glucosidase activity found in *Erwinia amylovora* contributes to bacterial virulence by allowing the bacteria to infect plants that contain β-glucosides without inducing the formation of toxic aglycones. To test this suggestion, we created strains of *E. amylovora* which had high β-glucosidase activities and studied the ability of these strains to cause fire blight disease in pears (*Pyrus communis*). We isolated spontaneous mutants that were able to utilize β-glucosides as the sole carbon source and showed that one class had about 10 times as much β-glucosidase activity as the wild-type strain. In addition, we constructed several plasmids that carry the *Escherichia coli bgl* operon under the control of a transposon Tn*5* promoter that is expressed in *E. amylovora*. These plasmids were introduced in *E. amylovora* by transformation. Pathogenesis studies in immature Bartlett pear fruits, etiolated sprouts, and young shoots showed that a 100-fold increase in β-glucosidase activity does not interfere with normal development of fire blight disease in these model systems.

The enteric bacterium *Erwinia amylovora* causes fire blight disease in a wide range of rosaceous plants, including pears (*Pyrus communis*) (28). Pear tissues infected by the bacteria develop a dark brown or black color which contributes to the charred appearance characteristic of the disease. The color results from the oxidation of phenolic compounds produced during the breakdown of arbutin, a phenolic β-glucoside found in pears. Arbutin is relatively nontoxic, but it can be hydrolyzed by β-glucosidase enzymes to release hydroquinone. Strains of *E. amylovora* in culture are typically inhibited by hydroquinone concentrations of 200 to 500 ppm (μg/ml) (3). Because the arbutin concentration in pears is approximately 10,000 ppm and free hydroquinone concentrations ranging up to 8,000 ppm have been reported in damaged pear tissue (27), several groups of investigators have suggested that hydroquinone plays a role in plant resistance to fire blight disease (3, 11, 27).

Hildebrand and co-workers demonstrated an antibacterial substance in pear blossoms (11), leaves (12), and xylem (10, 13) that was related to the hydroquinone released by degradation of arbutin. Antibacterial activity is correlated with hydroquinone concentration in aqueous extracts prepared from resistant and susceptible pear tissues (9). In addition, D-glucono-δ-lactone, a glucosidase inhibitor, simultaneously inhibits arbutin hydrolysis and the development of antibacterial activity in pear tissues (27).

The putative arbutin defense could be activated by plant β-glucosidases (9, 11–13), bacterial β-glucosidases (3, 14, 26), or both. If bacterial enzymes are involved, the low β-glucosidase activity of *E. amylovora* might contribute to bacterial virulence by permitting the bacteria to grow in plant tissues that contain β-glucose-linked antimetabolites (26).

To determine the role of β-glucoside antimetabolites in resistance to fire blight and to study the role of bacterial β-glucosidase activity in the pathogenesis of *E. amylovora*, we studied the virulence of isogenic *E. amylovora* strains that express different levels of β-glucosidase activity. Our results show that increasing the β-glucosidase activity does not prevent *E. amylovora* from causing fire blight in Bartlett pear fruit, seedlings, or sprouts.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1.

Media and chemicals. LB broth (22) was the complex medium used for growing both *Escherichia coli* and *E. amylovora*. M9 minimal medium (22) was used for *E. coli*. Minimal medium for *E. amylovora* contained 3 g of K_2HPO_4, 1 g of NaH_2PO_4, 1 g of NH_4Cl, 0.15 g of KCl, 0.1 g of $CaCl_2$, 0.3 g of $MgSO_4 \cdot 7H_2O$, 2.5 mg of $FeSO_4$, and 50 mg of nicotinic acid per liter supplemented with an appropriate carbon source at 0.5% (wt/vol). Agar was added at 1.5% for plates and 0.7% for overlays. When indicated, *E. coli* medium was supplemented with kanamycin sulfate at 75 μg/ml, tetracycline at 25 μg/ml, and ampicillin at 100 μg/ml; *E. amylovora* medium was supplemented with kanamycin sulfate at 50 μg/ml, tetracycline at 10 μg/ml, and ampicillin at 100 μg/ml. All antibiotics were from Sigma Chemical Co. (St. Louis, Mo.).

DNA purification, plasmid construction, and transformation. Plasmid DNA was isolated from *E. coli* and *E. amylovora* by the Triton X-100 lysis method (16). DNA manipulations, including restriction, ligation, and electrophoretic analysis, have been described previously (16). Restriction endonucleases and T4 DNA ligase were purchased from New England BioLabs, Inc. (Beverly, Mass.), and used under the conditions suggested by the manufacturer. Plasmid DNA was transformed into *E. amylovora* by a modification of the $CaCl_2$ method (20). Cells were grown in LB medium supplemented with 1 mM $MgCl_2$–0.5 mM $CaCl_2$, washed first with 100 mM $MgCl_2$ and then with 50 mM

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TABLE 1. Bacterial strains, phage, and plasmids used in this study

Bacteria and plasmids	Characteristics	Source or reference
Bacteria		
E. coli C-1a	Wild type	Our laboratory
E. amylovora		
Ea178	Wild type	A. K. Chatterjee (4)
Ea226	Wild type from pear; isolated in New York, 1977	S. V. Beer
Ea324	Wild type from hawthorne; isolated in France by Paulin, 1972	S. V. Beer
2027	Wild type from pear; Str^r; isolated in Washington	R. Covey
2028	Wild type from pear; Str^r; isolated in Washington	R. Covey
E9	Wild type from apple	A. K. Chatterjee (2)
Ea324-A	$Arbutin^+$, $salicin^+$ mutant of Ea324	This study
Ea324-C	$Cellobiose^+$ mutant of Ea324	This study
Plasmids		
pBR322	amp^r tet^r	19
pRZ102	kan^r Tn*5*	15
pAR6	From pBR322; *bglRCSB*, β-glucosidase null	24
pSAL6	From pBR322; *bglR* IS*1* *bglCBS* Bgl^+	24
pTK101	From pSAL6; *bglR* IS*1* *bglCBS* Bgl^+	This study
pTK105	From pTK101; *bglR* IS*1* *bglCBS* kan^r Bgl^+	This study
pTK110	From pTK101; *bglR* IS*1* *bglCBS* kan^r, β-glucosidase constitutive	This study

$CaCl_2$, and heat shocked at 42°C for 2 min. The cells were grown in LB broth for at least 3 h after transformation to allow expression of antibiotic resistance markers. The transformation frequency for different strains of *E. amylovora* ranged from 10^1 to 10^4 transformants per μg of DNA. Plasmid DNA that was reisolated from *E. amylovora* did not have any gross structural rearrangements, as determined by restriction enzyme analysis. The relatively low efficiency of transformation was not due to an *E. amylovora* restriction enzyme because plasmid DNA isolated from *E. amylovora* gave similar frequencies of transformation. Plasmids with either pMB1 or RK2 origins of replication were lost from *E. amylovora* at a frequency of about 2×10^{-3} per generation. Transformation of *E. amylovora* with pBR322 has been reported previously (19; D. W. Bauer and S. V. Beer, Phytopathology 73:1342, 1983).

Construction of plasmids carrying the *E. coli bgl* operon. The *E. coli bgl* operon has been cloned previously by Reynolds et al. (24) in both an inactive form (pAR6) and in a form activated by insertion of IS*1* into the *bglR* locus (pSAL6) (Fig. 1). Plasmid pTK101 (Fig. 1) carries a *bgl* operon in which some of the upstream regulatory region has been deleted. This deletion does not inactivate the induction of the *bgl* operon by salicin but does alter its sensitivity to glucose catabolite repression (see below). To increase β-glucosidase activity and to make expression constitutive, we inserted the Tn*5* kanamycin resistance gene with its promoter (15) into a site within the IS*1* sequence that is upstream of the *bgl* operon in pSAL6. In pTK105 the gene is read away from the *bgl* operon, while in pTK110 it is read toward the operon. These plasmid constructions therefore should lead to different levels of β-glucosidase expression in a host cell and have different regulatory properties in the presence of inducers and repressors of *bgl* gene expression. Activation of the *bgl* promoter is complex (24), and it should be noted that in addition to the kanamycin phosphotransferase promoter, some of the sequences that control normal expression of the *bgl* operon were retained in all of our constructions.

β-Glucosidase assays. β-Glucosidase activities were determined by measuring the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (PNPG) by intact cells (25). This is actually a coupled assay because degradation of β-glucosides by *E. coli* occurs by phosphorylation of the β-glucoside during transport followed by hydrolysis of the glucosidic bond by a cytoplasmic phospho-β-glucosidase to release *p*-nitrophenol (PNP) (5). The cultures were grown to the late logarithmic growth phase in minimal medium containing glycerol, di-

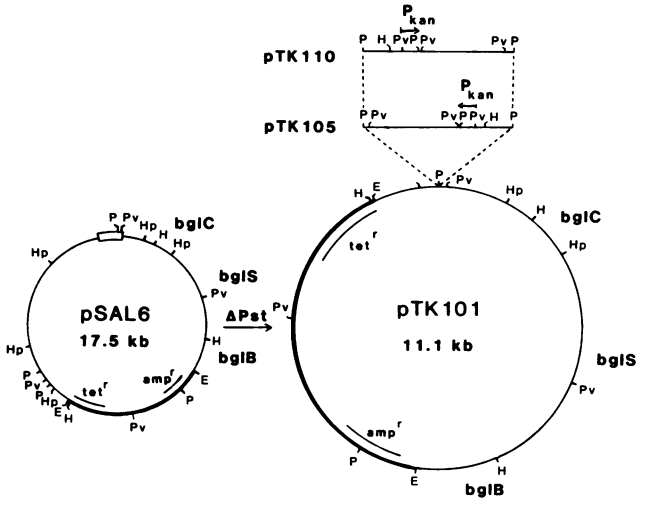

FIG. 1. Construction of plasmids with modified *bgl* operons. pTK101 was constructed by deleting the two *Pst*1 fragments upstream of the *bgl* operon of pSAL6 (24). In pTK105 and pTK110 these fragments were replaced by two *Pst*I fragments and isolated from pRZ102 (15), which carry the entire kanamycin phosphotransferase gene of Tn*5*. In pTK105 these fragments were inserted such that the kanamycin phosphotransferase promoter read in the opposite direction from the *bgl* operon, whereas in pTK110 it read toward the *bgl* operon. The boxed region in pSAL6 represents IS1. Enzyme abbreviations: E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; P, *Pst*I; Pv, *Pvu*II.

TABLE 2. β-Glucosidase activity of mutant and constructed *E. amylovora* strains

Strain	β-Glucosidase activity on the following carbon source[a]:					
	Salicin	Glycerol	Glucose	Salicin − glycerol	Salicin − glucose	Glycerol − glucose
Ea324	2.71 ± 0.70	1.91 ± 0.88	0.11 ± 0.15	1.74 ± 0.10	0.19 ± 0.14	0.14 ± 2.09
Ea324-C	4.35 ± 1.44	2.23 ± 1.28	0.45 ± 0.66	1.73 ± 1.93	0.31 ± 1.02	0.09 ± 1.71
Ea324-A	57.2 ± 3.3	82.4 ± 3.3	4.07 ± 0.69	58.4 ± 10.6	2.95 ± 0.83	3.20 ± 1.54
Ea324(pBR322)	1.56 ± 0.11	1.35 ± 2.08	0.09 ± 0.47	1.04 ± 0.57	0.18 ± 0.47	0.07 ± 0.06
Ea324(pAR6)	19.1 ± 2.2	29.1 ± 5.5	0.05 ± 0.11	33.3 ± 7.6	0.07 ± 0.20	0.21 ± 0.30
Ea324(pSAL6)	148 ± 23	91.3 ± 6.0	1.32 ± 0.34	180 ± 11	1.65 ± 0.41	0.53 ± 0.87
Ea324(pTK101)	210 ± 33	197 ± 14	4.25 ± 0.27	192 ± 10	9.87 ± 1.01	4.75 ± 0.50
Ea324(pTK105)	129 ± 32	115 ± 28	1.36 ± 0.17	165 ± 11	2.39 ± 0.41	1.02 ± 2.43
Ea324(pTK110)	166 ± 33	223 ± 30	10.5 ± 2.8	184 ± 5	33.3 ± 15.1	4.00 ± 1.13

[a] All strains were grown in glycerol minimal medium and shifted to the indicated carbon source. Each entry represents the mean ± standard deviation of four experiments. β-Glucosidase activities are expressed as nanomoles of PNP liberated per minute, normalized to 10^8 cells.

luted in minimal medium supplemented with 0.5% (wt/vol) of the indicated carbon source to an optical density at 600 nm of 0.2, and grown for an additional 2 h (*E. coli*) or 3 h (*E. amylovora*) to adapt to the expression medium.

To determine the level of β-glucosidase activity expressed during pathogenesis, bacteria were rinsed from the surface of infected pears, washed in saline, and adjusted to an A_{600} of 0.5. The reaction was started by the addition of PNPG to a final concentration of 2 mM, and the cells were incubated at 30°C. The reaction was stopped by the addition of Na_2CO_3 to a concentration of 0.5 M. The cells were lysed by adding sodium *N*-lauroyl sarcosine to 0.1%, and the liberated PNP was measured spectrophotometrically by determining the absorption at 415 nm with correction for light scattering measured at 540 nm. One unit of β-glucosidase activity is defined as the amount of enzyme that causes the release of 1 nmol of PNP in 1 min. All enzyme activities were determined in quadruplicate and normalized to a culture density of 10^8 cells per ml.

To verify that the more-sensitive PNPG hydrolysis assay represented an activity that could hydrolyze arbutin, the production of hydroquinone by cells grown in arbutin was estimated by measuring the A_{540} of polymerized phenolic compounds after overnight exposure to air and by relating this to standards prepared with known amounts of hydroquinone. No polymerization of unhydrolyzed arbutin was observed under these conditions.

Pathogenicity tests of *E. amylovora*. Immature Bartlett pears (diameter, 1.5 to 2.0 cm) were surface sterilized by immersion in 1% sodium hypochlorite for 15 min and air dried. Each pear was inoculated by wounding the outer skin with a toothpick that had been touched to a fresh *E. amylovora* colony on LB agar. The pears were placed in sterile test tubes and incubated in moisture chambers at room temperature. Etiolated pear sprouts (length, 3.5 to 4.5 cm) and the green shoots of young potted plants (height, 10 to 15 cm) (21) were inoculated by wounding the epidermis with a sterile 20-gauge needle and placing a 10-μl drop of inoculant on the wound. Bacteria used in dose-response experiments were grown to the middle logarithmic growth phase in glycerol minimal medium and diluted in sterile saline. The bacterial challenge was varied from 10^4 to 10^8 cells in experiments with immature pear fruit and from 10^1 to 10^7 cells in experiments with etiolated sprouts and young shoots. All pathogenicity tests were done with at least four replicate samples and scored at frequent intervals for symptoms of disease such as tissue necrosis, tissue blackening, and the oozing of polysaccharides from the tissue.

RESULTS

Spontaneous β-glucoside-utilizing mutants of *E. amylovora*. Wild-type strains of *E. amylovora* have very low β-glucosidase activity (14, 26). To study the relationship between β-glucosidase activity in *E. amylovora* and bacterial pathogenicity in pears, we used two different methods to increase the bacterial β-glucosidase activity. In the first method, we isolated spontaneous mutants that could utilize a β-glucoside as sole carbon source. In the second method, the *bgl* operon from *E. coli* was cloned into a multicopy number plasmid downstream from a promoter for the Tn*5* kanamycin resistance gene, and the plasmid was then transformed into *E. amylovora*.

The six wild-type strains that we tested all grew very slowly on media containing a β-glucoside (salicin, arbutin, cellobiose, amygdalin, or esculin) as the carbon source. In an attempt to isolate spontaneous mutants with higher β-glucosidase activities, we plated 10^8 wild-type cells of the strains listed in Table 1 on media containing one of these β-glucosides and isolated mutants that grew rapidly. The growth of each mutant was then tested on the other β-glucosides. We found no mutants that could grow on amygdalin or esculin. The ability to grow on arbutin and salicin often arose simultaneously, independent of which medium was first used for selection. Mutants able to grow on cellobiose did not gain the ability to grow on other β-glucosides.

The β-glucosidase activity of many of these mutants was measured; and two mutants of *E. amylovora* Ea324, Ea324-A, and Ea324-C were taken as representative of the arbutin$^+$ and cellobiose$^+$ classes of mutants (Table 2). Mutant Ea324-A had about 10 times the β-glucosidase activity of the wild-type Ea324, but strain Ea324-C had only slightly higher β-glucosidase activity than Ea324. The low-level β-glucosidase activity of wild-type *E. amylovora* Ea324 was partially repressed by the presence of glucose. The β-glucosidase activity of Ea324-A was not dependent on salicin for induction, but was repressed by glucose.

Because the mode of resistance to fire blight disease has been proposed to involve the toxicity of liberated hydroquinone to the pathogen (3, 26), the sensitivity of the mutant strains to hydroquinone was determined by streaking them on minimal glucose plates supplemented with 1 to 1,000 μg of hydroquinone per ml. No changes in the MICs of 200 to 500 μg/ml were observed for any of the mutant strains.

Expression of plasmid-borne *bgl* operons in *E. coli*. The second method we used to raise the level of β-glucosidase

TABLE 3. β-Glucosidase activity of *E. coli* carrying constructed plasmids

Strain	β-Glucosidase activity on the following carbon source[a]:					
	Salicin	Glycerol	Glucose	Salicin − glycerol	Salicin − glucose	Glycerol − glucose
C-la	0.16 ± 0.15	0.58 ± 0.55	0.88 ± 0.28	0.34 ± 0.28	0.55 ± 0.23	0.69 ± 0.16
C-la(pBR322)	0.22 ± 0.09	0.32 ± 0.19	1.09 ± 0.21	0.24 ± 0.18	1.13 ± 0.79	0.72 ± 0.25
C-la(pAR6)	144 ± 86	80.1 ± 22.8	39.6 ± 4.9	62.7 ± 11.3	86.6 ± 28.7	64.2 ± 36.0
C-la(pSAL6)	536 ± 427	121 ± 143	176 ± 164	698 ± 286	240 ± 123	296 ± 76.3
C-la(pTK101)	1,000 ± 370	201 ± 232	292 ± 265	1,120 ± 260	1,060 ± 420	156 ± 138
C-la(pTK105)	177 ± 67	157 ± 97	194 ± 31	339 ± 140	173 ± 161	210 ± 144
C-la(pTK110)	497 ± 169	461 ± 164	678 ± 229	1,150 ± 270	516 ± 229	1,260 ± 840

[a] All strains were grown in glycerol minimal medium and shifted to the indicated carbon source. Each entry represents the mean ± standard deviation of four experiments. β-Glucosidase activities are expressed as nanomoles of PNP liberated per minute, normalized to 10^8 cells.

expression was to clone the *E. coli bgl* operon into a multicopy number plasmid downstream from promoters expressed in *E. amylovora*. To determine the regulation and level of expression of the *bgl* operon on each of the constructed plasmids, we transformed the plasmids into *E. coli* C-1a and assayed β-glucosidase activity during growth on several carbon sources after transfer from glycerol medium (Table 3). Wild-type *E. coli* C-1a had a very low β-glucosidase activity. The wild-type *bgl* operon on pAR6 gave a moderate level of β-glucosidase activity. The activated, but otherwise unaltered, operon on pSAL6 expressed an inducible β-glucosidase activity that was partially repressed in the presence of glucose. Deletion of the regulatory region of the *bgl* operon on pTK101 eliminated repression of gene activity by glucose and enhanced the expression of the operon in the presence of inducer. In pTK105 the promoter of the kanamycin phosphotransferase gene reads in the opposite orientation from the *bgl* operon, and β-glucosidase activity in the presence of salicin was much lower than that of the parent plasmid pTK101. In pTK110 the same promoter reads toward the *bgl* operon. β-Glucosidase expression by pTK110 did not require the presence of β-glucoside inducers and was not inhibited by the presence of glucose. Thus, in *E. coli* the operon carried on pTK101 was inducible by salicin and independent of glucose catabolite repression, the operon carried by pTK105 was uninducible, and the operon carried by pTK110 was constitutive with a high level of expression.

Expression of plasmid-borne *bgl* operons in *E. amylovora*. Each of the recombinant plasmids was transformed into *E. amylovora* Ea324 to determine the level of expression and mode of regulation of the *bgl* operons. All plasmid-containing strains grew on salicin and also grew, although slowly, on arbutin. The absolute levels of β-glucosidase activity for the constructed plasmids in *E. amylovora* averaged about 20% of the corresponding activity in *E. coli* (Table 2). Strain Ea324(pBR322) had low β-glucosidase activity in all media. Salicin induced β-glucosidase activity in Ea324(pSAL6) but had no effect on activity in the other plasmid-containing strains. Interestingly, a strong glucose catabolite repression was observed in all strains, although pTK110 was least affected. The β-glucosidase activity expressed by strains carrying pTK110 was approximately 100 times higher than that of wild-type *E. amylovora* on all media.

To demonstrate that the *bgl* operon on the constructed plasmids was expressed in the presence of arbutin and that PNPG-hydrolyzing activity was a good estimator of the ability to hydrolyze arbutin, we grew the constructed *E. amylovora* strains using arbutin as the sole carbon source and measured the PNPG-hydrolyzing and hydroquinone-liberating activities (Table 4). All strains that expressed β-glucosidase activity when grown in salicin medium (Table 2) also had high activity when grown in arbutin medium (Table 4). The data in Table 4 also show that PNPG-hydrolyzing activity is related to the ability to liberate hydroquinone from arbutin.

Pathogenicity of *E. amylovora* strains with increased β-glucosidase activity. The pathogenicity of the mutant or constructed strains was tested by inoculating the bacteria into immature fruit, etiolated sprouts, and young shoots of Bartlett pears (21). We found no significant differences between wild-type *E. amylovora* Ea324 and any of the mutant or constructed strains in the minimal dose required for disease to develop, the time until symptoms were first observed, the progression of the disease, or the severity of the symptoms. Representative data, using immature pears as host tissue, are shown in Table 5. In tests with etiolated sprouts, necrosis of a 1-cm length of the sprout was observed after 26 to 43 h, when as few as 10^1 cells were used as inoculum, irrespective of the genotype of the pathogen. When green shoots were inoculated, oozing from the wound or from a second site on the stem was observed after 40 to 80 h, followed by blackening of the stem and leaves and the eventual death of the plant. The dose response of green shoots was more variable; generally, between 10^3 and 10^5 cells were required to cause disease, although in some cases a smaller inoculum resulted in later appearance of the disease.

TABLE 4. β-Glucosidase activity of *E. amylovora* during pathogenesis and growth on arbutin

Strain	β-Glucosidase activity on the following nutrient sources determined by the indicated assay:		
	Pear (PNPG)[a]	Arbutin[b]	
		PNPG	Hydroquinone
Ea324	4.19 ± 0.33	3.98 ± 0.15	168 ± 48
Ea324-C	3.52 ± 0.16	4.38 ± 0.36	74 ± 26
Ea324-A	24.2 ± 0.3	91.5 ± 4.2	564 ± 7
Ea324(pBR322)	1.29 ± 0.14	5.93 ± 0.76	129 ± 106
Ea324(pAR6)	68.6 ± 1.6	57.5 ± 4.9	1,020 ± 76
Ea324(pSAL6)	174 ± 2	86.4 ± 16.9	1,790 ± 44
Ea324(pTK101)	220 ± 2	99.9 ± 2.3	2,310 ± 87
Ea324(pTK105)	167 ± 1	69.2 ± 8.4	1,720 ± 112
Ea324(pTK110)	211 ± 5	134 ± 5	1,720 ± 25

[a] Bacteria were isolated from the surface of infected pears.

[b] Strains were grown in glycerol minimal medium and shifted to arbutin. β-Glucosidase activities are expressed as nanomoles of PNP liberated per minute. Oxidized hydroquinone was measured as described in the text. All activities were normalized to 10^8 cells. Each entry represents the mean ± standard deviation of four experiments.

To determine whether the bacteria that caused disease had the same properties as those that were inoculated into the plants, we reisolated bacteria from the pear surface opposite from the site of inoculation by using nonselective LB medium. These bacteria were analyzed by their growth characteristics on various carbon sources and were tested for maintenance of the plasmids by determining whether they could grow in the presence of kanamycin or tetracycline. No revertants were observed, and all plasmids were maintained throughout the infection. The β-glucosidase activity of these reisolated bacteria was determined in an experiment similar to that for which the results are shown in Table 2, and the original pattern of β-glucosidase activities was observed (data not shown).

To determine the level of β-glucosidase activity expressed during pathogenesis, bacteria were rinsed from the surface of inoculated pears after considerable ooze had formed, washed twice in saline to remove plant-derived β-glucosidase, and assayed for β-glucosidase activity (Table 4). Bacteria that were rinsed from pears that had been inoculated with wild-type *E. amylovora* or strains with plasmids that did not carry a *bgl* operon had low levels of β-glucosidase activity. These activities were higher, however, than those observed ex planta. We do not know whether this increase in activity was due to contamination by plant enzymes or to induction of bacterial enzymes in the plant tissue. All strains that had high levels of β-glucosidase when assayed in the presence of β-glucosidase inducers (Table 2) also had high levels in these assays of plant-derived material. These results demonstrate that β-glucosidase activity is expressed by *E. amylovora* in the plant tissue but that this activity does not interfere with the normal development of fire blight disease.

TABLE 5. Pathogenesis of *E. amylovora* strains on immature pear fruit

Strain	Time (h) to reach 1 cm[a]
Ea324	48.5 ± 5.9
Ea324-C	41.6 ± 1.9
Ea324-A	48.5 ± 3.6
Ea324(pBR322)	51.0 ± 5.2
Ea324(pAR6)	52.0 ± 10.9
Ea324(pSAL6)	51.5 ± 6.2
Ea324(pTK101)	54.5 ± 4.8
Ea324(pTK105)	51.6 ± 4.0
Ea324(pTK110)	47.8 ± 2.6

[a] Mean time ± standard deviation (for six replicate samples) required for necrotic tissue to reach 1 cm in diameter.

DISCUSSION

Plant tissues contain various metabolic inhibitors that are thought to be important in defense against bacterial and fungal pathogens (1). To prevent these antimetabolites from interfering with normal plant metabolism, they must either be compartmentalized in specialized storage structures or modified chemically so that they are nontoxic until converted to their active form. The most common modification of antimetabolites is glycosylation (1, 17). After infection by a pathogen, the toxic aglycones can be released by host or pathogen glycosidase enzymes (1, 9, 17) and may retard the spread of the infection.

Pears contain high concentrations of arbutin, a β-glucoside that releases hydroquinone after hydrolysis. It has been suggested (3, 26) that the low level of β-glucosidase expressed by *E. amylovora* fails to activate the arbutin chemical defense and that this characteristic contributes to bacterial virulence. If this hypothesis were correct, we would expect that increased bacterial β-glucosidase levels would decrease bacterial virulence. Using two different methods, we constructed *E. amylovora* strains that produce high levels of β-glucosidase. In contrast to the prediction, these strains cause disease that is indistinguishable from that caused by the wild-type parent strain. Even though we did not study pathogenesis in blossoms (the entry tissue in many fire blight disease infections) or wounds on mature, growing trees, the three different virulence assays used in these experiments should be a reliable measure of virulence. Our results show no relationship between the ability of *E. amylovora* to hydrolyze arbutin and its ability to cause fire blight disease in pears. They also call into question the effectiveness of hydroquinone produced from arbutin as a defense against *E. amylovora*, no matter what enzyme is proposed to be responsible for its production. The direct oxidation products of arbutin (8, 23) may play a role in plant defense, but our experiments did not test the effect of these compounds.

The β-glucosidase activities we measured in our mutant and constructed strains were comparable to those seen in epiphytic *Erwinia herbicola* and *Pseudomonas* bacteria that are found associated with the same plants attacked by *E. amylovora* (3, 26). *E. herbicola* has been postulated to be avirulent because its β-glucosidase activities are higher than those of *E. amylovora* (26). *Pseudomonas syringae*, in contrast to *E. amylovora*, is tolerant of hydroquinone, but it can cause only a limited infection of pear tissue (26). The β-glucosidase produced by our *E. amylovora* strains was sufficient to allow them to grow rapidly by using β-glucosides as their sole source of carbon. When grown on media that contained arbutin, these strains liberated substantial quantities of hydroquinone. Our bacterial strains retained the ability to produce β-glucosidase during the course of infection, and we have shown that they make a substantial contribution to the level of β-glucosidase present in exudates isolated from infected tissues. Thus, the enzymatic activity was present but it was not effective in limiting the disease.

Although we interpret our data to mean that the lack of an active β-glucosidase enzyme in *E. amylovora* is not essential for pathogenesis, it is possible that strains with high β-glucosidase activities would not compete successfully with strains that had low activities, even though both cause disease. If so, this competitive advantage would explain the low level of β-glucosidase usually found in *E. amylovora*. In this regard, the *E. amylovora-P. communis* interaction may be similar to the interaction between *Erwinia chrysanthemi* and corn (*Zea mays*). In *E. chrysanthemi*, resistance to the plant phenolic compound 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one is found primarily in strains isolated from corn, bacterial resistance is not essential to the development of disease (18). It is also possible that *E. amylovora* strains with high β-glucosidase levels will be avirulent in a host defended by a different set of β-glucosides.

Because we were able to select *E. amylovora* strains that could grow on various β-glucosides, it seems likely that the catabolic genes are present in *E. amylovora* but are not expressed. β-glucosidase activity is cryptic in many bacteria (7), and to explain this Hall et al. (7) have suggested that the β-glucoside phosphates formed during catabolism can accumulate and inhibit growth. When cellobiose (glucose-β-D-glucose) is added to cells growing on glycerol as carbon

source, Cel$^+$ strains are inhibited but cellobiose-null strains are not (6). Thus, the ability to catabolize β-glucosides may sometimes be disadvantageous, irrespective of whether toxic aglycones are released by hydrolysis. *E. amylovora* populations drastically decrease during the winter, and the bacteria that survive in infected trees are the source of new infections when spring returns (28). Because the tissues in which overwintering occurs contain arbutin, a selection for low β-glucosidase activity could occur at this stage of the disease cycle.

In most of the studies in which it has been suggested that arbutin hydrolysis is a factor in resistance to fire blight disease, excised tissues and tissue homogenates or extracts (9–13, 27) were used. These treatments can induce a number of resistance mechanisms and may mix enzymes and substrates that would normally be compartmentalized. In this study we genetically altered the metabolism of *E. amylovora* to study the effect of these alterations on pathogenesis in relatively undisturbed plant tissues and in whole plants. This strategy was practical because of the development of a transformation protocol for *E. amylovora* and because of the availability of genes in the closely related bacterium *E. coli* that could be expressed in *E. amylovora*. Our results show that while expression of *E. coli* genes may be altered in *E. amylovora*, genetic manipulation of this pathogen may nevertheless be useful in testing models of its virulence mechanisms.

ACKNOWLEDGMENTS

We thank Barry Hall for helpful discussions; A. K. Chatterjee, R. P. Covey, S. V. Beer, and A. Wright for strains; and Linda R. Moore for critically reading the manuscript.

Part of this study was supported by a grant from the Washington Tree Fruit Commission.

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