Pantoea agglomerans Strain EH318 Produces Two Antibiotics That Inhibit Erwinia amylovora In Vitro

SANDRA A. I. WRIGHT,† CATHY H. ZUMOFF, LOIS SCHNEIDER, AND STEVEN V. BEER*

Department of Plant Pathology, Cornell University, Ithaca, New York 14853

Received 31 May 2000/Accepted 3 October 2000

Pantoea agglomerans (synonym: Erwinia herbicola) strain EH318 produces through antibiosis a complex zone of inhibited growth in an overlay seeded with Erwinia amylovora, the causal agent of fire blight. This zone is caused by two antibiotics, named pantocin A and B. Using a genomic library of Eh318, two cosmids, pCPP702 and pCPP704, were identified that conferred on Escherichia coli the ability to inhibit growth of E. amylovora. The two cosmids conferred different antibiotic activities on E. coli DH5α and had distinct restriction enzyme profiles. A smaller, antibiotic-conferring DNA segment from each cosmid was cloned. Each subclone was characterized and mutagenized with transposons to generate clones that were deficient in conferring pantocin A and B production, respectively. Mutated subclones were introduced into Eh318 to create three antibiotic-defective marker exchange mutants: strain Eh421 (pantocin A deficient); strain Eh439 (pantocin B deficient), and Eh440 (deficient in both pantocins). Cross-hybridization results, restriction maps, and spectrum-of-activity data using the subclones and marker exchange mutants, supported the presence of two distinct antibiotics, pantocin A and pantocin B, whose biosynthetic genes were present in pCPP702 and pCPP704, respectively. The structure of pantocin A is unknown, whereas that of pantocin B has been determined as (R)-N-[(S)-2-amino-propanoyl(l)-amino]-methyl]-2-methanesulfonyl-succinamic acid. The two pantocins mainly affect other enteric bacteria, based on limited testing.

Pantoea agglomerans or Pantoea dispersa (20), also known as Erwinia herbicola (Löhnis) Dye are members of the Enterobacteriaceae and are ubiquitous in nature, inhabiting plants, soil, and water (16, 20, 21) and animals and humans (16, 35). Strains belonging to E. herbicola are members of the E. herbicola-Enterobacter agglomerans cluster; some have been redesignated P. agglomerans and P. dispersa, while others did not fall into either of the two species (20). P. agglomerans and P. dispersa are frequent companions of E. herbicola-Enterobacter agglomerans cluster; some have been redesignated P. agglomerans and P. dispersa as biological control agents for fire blight of apple and pear trees (36, 38). There is current interest in P. agglomerans and P. dispersa as biological control agents for fire blight because they are harmless to apple and pear trees and are able to protect them against invasion of the pathogen (4, 29). P. agglomerans strain EH318, isolated from a symptomless apple stem in New York State, protected immature pear fruits in the orchard tests (5, 23, 43).

Production of antibiotics inhibitory to E. amylovora by several strains of Pantoea spp. seems important for inhibition of E. amylovora in planta (30, 45, 53). In vitro inhibition of E. amylovora by antibiotics of Pantoea spp. is well documented (24, 28, 45, 47, 48). Different strains of P. agglomerans and P. dispersa have different spectra of antimicrobial activity (15, 25) and produce different types of inhibition zones against the same indicator organism (3); both observations presumably reflect the fact that different antibiotics are produced by different strains. One strain of P. agglomerans, strain C9-1, produces three different antibiotics, which were purified and characterized preliminarily (27). The presence of an inner and an outer zone of inhibition in an E. amylovora 110-seeded agar overlay led Ishimaru and coworkers to suggest that P. agglomerans C9-1 produced more than one antibiotic (28). P. agglomerans Eh318 also forms a double halo in an overlay seeded with E. amylovora strain Ea273 (Fig. 1), which we hypothesized was due to the action of two antibiotics (S. Wright-Dobrzeniecka and S. V. Beer, Abstr. 93rd Gen. Meet. Am. Soc. Microbiol. 1993, abstr. Q420, 1993). One of these, pantocin B has been characterized chemically as (R)-N-[(S)-2-amino-propanoyl(l)-amino]-methyl]-2-methanesulfonyl-succinamic acid (10); it inhibits N-acetylornithine transaminase through competitive binding with N-acetylornithine, thus interfering with the last step in the arginine biosynthetic pathway (10, 49). Antibiotics of Pantoea species frequently are grouped on the basis of the type of amino acid that, when added to the overlay, renders E. amylovora insensitive to them. Most strains of P. agglomerans and P. dispersa produce histidine-reversible or histidine- and/or leucine-reversible antibiotics (14, 50). The biosynthesis of E. amylovora by P. agglomerans Eh318 is abolished in the presence of a combination of histidine and arginine, but not by either amino acid alone (48, 49).

We have demonstrated that two distinct cosmids, pCPP702 and pCPP704, containing inserts of Eh318 DNA bestow on E. coli the ability to produce two distinct antibiotics inhibitory to E. amylovora. The observed requirement for two amino acids to abolish antibiosis by Eh318 is a consequence of the two antibiotics. They were named pantocins after the genus name of the producing organism. Histidine reversed the activity of pantocin A, and arginine reversed that of pantocin B. The distinctive antibiotic phenotypes of defined marker-exchange mutants of Eh318 that are defective in production of pantocin

* Corresponding author. Mailing address: Department of Plant Pathology, Cornell University, Ithaca, NY 14853. Phone: (607) 255-7878. Fax: (607) 255-4471. E-mail: svbii@cornell.edu.

† Present address: Plant Pathology and Biocontrol Unit, SLU, 750 07 Uppsala, Sweden.
acids were added alone. These concentrations were halved when the amino acids concentration of 0.76 g/ml and histidine was added at 1 g/ml, when the amino acids tinely were incubated for 2 days to allow for antibiotic production. When deter-

FIG. 1. *P. agglomerans* Eh318 produces a double zone of inhibition against *E. amylovora* Ea273 in a chloroform assay.

A and/or B provide clear genetic evidence for the production of these two antibiotics by *P. agglomerans.*

(Brief reports on these findings were made previously at scientific conferences [56; Wright-Dobrzeniecka and Beer, Abstr. 93rd Gen. Meet. Am. Soc. Microbiol 1993].)

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All strains were cultured routinely in Luria-Bertani (LB) medium (39). The reaction of strains Eh318 and Eh252 (Table 1) in API 20E (bioMérieux Vitek, Inc., Hazelwood, Mo.) was consistent with their identification as species of *Pantoea.* The results of a GN2 Microlog test (Biolog, Inc., Hayward, Calif.) identified them with highest probability as belonging to *P. agglomerans.* *E. amylovora* and *Pantoea* spp. were cultured at 28°C, and *Escherichia coli* was cultured at 37°C. The following antibi-

Antibiotic production assays were done on minimal media, either glucose-asparagine (GA) medium (52, 53) or *E. coli* minimal medium (EcMM), which contained per liter: 0.25 g of yeast extract (Difco Laboratories, Detroit, Mich.), 20 ml of glycerol, 4.0 g of K$_2$HPO$_4$, 1.72 g of KH$_2$PO$_4$, 0.5 g of NaCl, 2.0 g of (NH$_4$)$_2$SO$_4$, 0.2 g of sodium citrate, and 0.02 g of MgSO$_4$·7H$_2$O. Thiamine was added to GA medium and EcMM at 0.1 μg/ml for the growth of *E. coli* strains DH5α and JM109.

Antibiotic production assays. Antibiotic production was assayed by two meth-

ods, the live assay and the chloroform assay. In both assays, a basal layer of either GA medium or EcMM, which contained per liter: 0.25 g of yeast extract (Difco Laboratories, Detroit, Mich.), 20 ml of glycerol, 4.0 g of K$_2$HPO$_4$, 1.72 g of KH$_2$PO$_4$, 0.5 g of NaCl, 2.0 g of (NH$_4$)$_2$SO$_4$, 0.2 g of sodium citrate, and 0.02 g of MgSO$_4$·7H$_2$O. Thiamine was added to GA medium and EcMM at 0.1 μg/ml for the growth of *E. coli* strains DH5α and JM109.

DNA isolation and manipulations. For the construction of a genomic library, the DNA from plasmid pCPP9 (2) was isolated by the large-scale alkaline lysis procedure (39) and purified further by cesium chloride-ethidium bromide equi-

librium centrifugation (39). Isolation of total genomic DNA of Eh318 and its mutants, whether for library construction or to run on gels for Southern blotting, followed the procedure of Slikhuy and coworkers (40). Cosmid DNA was isolated by a medium-scale alkaline plasmid preparation procedure that combined the methods described by Marko et al. (33) and Zasloff et al. (57). Plasmid DNA was routinely isolated on a small scale by an alkaline miniprep extraction procedure (7).

Restriction endonucleases were purchased from Promega Corp. (Madison, Wis.), and digestion of DNA was carried out as recommended by the manufac-

Aicient intestinal alkaline phosphatase was obtained from Boehringer Mann-

hein (Indianapolis, Ind.) and DNA T4 ligase was obtained from Bethesda Research Laboratories (GIBCO BRL, Gaithersburg, Md.). Dephosphorylations and ligations followed standard procedures (39).

DNA in agarose gels for Southern transfer was depurinated, denatured, neutral-

ized, and transferred to Gene Screen Plus nylon membranes (Dupont, NEN Research Products, Boston, Mass.) according to the capillary blotting procedure suggested by the manufacturer (1). Prehybridization, hybridization and washes of membranes were done at 65°C and followed the protocol of Sambrook et al. (39), with the addition of 2.5 mM EDTA, 50 mM Tris-HCl (pH 8.0), and 10% polyethylene glycol to the prehybridization solution. Membranes were washed for 15 min in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.2% sodium dodecyl sulfate and twice in 0.4× SSC with 0.2% sodium dodecyl sulfate. The DNA fragment to be used as a probe was purified from agarose with the GeneClean Kit (Bio 101, Inc., La Jolla, Calif.) and labeled with 50 μCi of [α-32P]dGTP (Dupont, NEN) by the random primer labeling method (17). The probe was purified with a Sephadex G-50 spin column (Boehringer Mannheim). The membrane was exposed to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, N.Y.) at −80°C with a Cronex Lightning Plus intensifying screen (Eastman Kodak Co., Rochester, N.Y.).

Construction of a genomic library. Cloning of genomic DNA fragments of Eh318 of approximately 40 kb into the cosmid vector pCPP9 (2) followed the procedure of Ish-Horowicz and Burke (26), with the inclusion of a DNA sizing step on a 10 to 40% sucrose gradient (39). Fragments containing frames of 32 to 47 kb from a partial Sau3AI digest were dephosphorylated with calf intestinal alkaline phosphatase. The vector pCPP9 was digested separately with either EcoRI or SalI, further digested with BamHI, and ligated with the genomic Sau3AI fragments. Recombinant cosmids were packaged in vitro with the Giga-

packaging kit (Stratagene, La Jolla, Calif.), and transduced into *E. coli* JM109. Transductants were selected on the basis of their thionocyanin resis-

Screening the genomic library for antibiotic-producing transductants. Transductants were screened for antibiotic production in the live assay using an overlay seeded with Ea273. The cosmid DNA of colonies that produced antibi-

otics was extracted, digested with several restriction enzymes, and electropho-

resed to visualize restriction enzyme profiles. Antibiotics toward Ea273 in the presence and absence of arginine and histidine was evaluated by a modified chloroform assay in which the producer was allowed to grow for 2 days on GA medium before it was removed and the plate was exposed to chloroform vapors. DH5α carrying two distinct cosmids that conferred antibiotic toward Ea273 was assayed for activity against a number of different bacteria (see Table 2). The marker exchange mutants of Eh318 that were deficient in pantocin A and/or B synthesis (see below) were included to confirm that the activities of pantocin A and B were consistent in different genetic backgrounds. The results were re-

Downloaded from http://aem.asm.org/ on October 30, 2017 by guest
TABLE 1. Bacterial strains, phages, cosmids, and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain, phage, cosmid or plasmid</th>
<th>Relevant characteristics&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reference or source&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM109</td>
<td>recA1 thiA (lac-proAB) lacZΔM15</td>
<td>39</td>
</tr>
<tr>
<td>HB101</td>
<td>Sm&lt;sup&gt;r&lt;/sup&gt; recA1</td>
<td>39</td>
</tr>
<tr>
<td>DH5β</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; recA1 lacZΔM15 thi-1 Na&lt;sup&gt;r&lt;/sup&gt;</td>
<td>39</td>
</tr>
<tr>
<td>LE392</td>
<td>hsdR514 supE44 supF58 lacY1 gaiK2 galT22 metB1 trpR55 λ&lt;sup&gt;−&lt;/sup&gt;</td>
<td>39</td>
</tr>
<tr>
<td>CC118</td>
<td>Δ(ara-leu) 7697 ΔlacX74 ΔphoA20 galE gaiK thi rpsE spoB argE recA1 Sp&lt;sup&gt;i&lt;/sup&gt;</td>
<td>32</td>
</tr>
<tr>
<td>SM10(k&lt;sup&gt;pir&lt;/sup&gt;)</td>
<td>thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu Km&lt;sup&gt;r&lt;/sup&gt; kpir</td>
<td>34</td>
</tr>
<tr>
<td>CGSC0151</td>
<td>LamB204; resistant to lambda</td>
<td></td>
</tr>
<tr>
<td><strong>Erwinia amylovora</strong> Ea273</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pantoea agglomerans</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eh318</td>
<td>Rp&lt;sup&gt;i&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Eh421 (PanA&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>Marker exchange mutant of Eh318, deficient in pantocin A; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Rundle, CUCPB 2140, from apple leaves</td>
</tr>
<tr>
<td>Eh439 (PanB&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>Marker exchange mutant of Eh318, deficient in pantocin B; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work, CUCPB 4189</td>
</tr>
<tr>
<td>Eh440 (PanAB&lt;sup&gt;−&lt;/sup&gt;)</td>
<td>Marker exchange mutant of Eh318, deficient in pantocin A and B; Km&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This work, CUCPB 4433</td>
</tr>
<tr>
<td>Eh252</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pantoea sp. Eh112Y</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P. stewartii</strong> 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Erwinia carotovora</strong> subsp. carotovora B12</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Erwinia carotovora</strong> subsp. betavasculorum 101</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Erwinia chrysanthemi</strong> 3665</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Enterobacter aerogenes</strong> 1422/77</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Serratia marcescens</strong> Ser 101</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Klebsiella pneumoniae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pseudomonas putida</strong> W713</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pseudomonas syringae pv. tomato DC3000</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Xanthomonas campestris</strong> pv. pelargonii Xpel-1</td>
<td>Geranium isolate</td>
<td>M. Daughtery, CUCPB 4672</td>
</tr>
<tr>
<td><strong>Agrobacterium tumefaciens</strong> C58</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Phage A467</strong></td>
<td>λb221 rec::Tn5 cI857 Oam29 Pam80 Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>6</td>
</tr>
<tr>
<td><strong>Cosmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCPP702</td>
<td>Sp&lt;sup&gt;i&lt;/sup&gt; Sm&lt;sup&gt;r&lt;/sup&gt;; contains genomic fragments cloned in pCPP9, responsible for synthesis of pantocin A</td>
<td>This work</td>
</tr>
<tr>
<td>pCPP704</td>
<td>Sp&lt;sup&gt;i&lt;/sup&gt; Sm&lt;sup&gt;r&lt;/sup&gt;; contains genomic fragments cloned in pCPP9, responsible for synthesis of pantocin B</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCPP9</td>
<td>pGB2-mob λcos Sp&lt;sup&gt;i&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>prRK2013</td>
<td>helper plasmid; Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>18</td>
</tr>
<tr>
<td>pBluescript KS(+)</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;; cloning vehicle</td>
<td>Stratagene Corp.</td>
</tr>
<tr>
<td>pCPP1051</td>
<td>8.4 and 11.8-kb EcoRI fragments of pCPP702 in pBluescript KS(+)</td>
<td>This work</td>
</tr>
</tbody>
</table>

Continued on following page
corded qualitatively (absence or presence of zones of inhibition) rather than quantitatively, since both the live and the chloroform tests were used. The sensitivities of Eh252 and Eh318 (control) to pantocin A and pantocin B produced by DH5a(pCPP1051) and DH5a(pCPP719) were tested separately. A colony of Ea273 that appeared in a zone of inhibition produced by Eh421 (deficient in pantocin A synthesis [PanA−]) was propagated several times on fresh plates where Eh421 had grown, in order to select for maintenance of the antibiotic resistance phenotype. The strain Ea273R421 was subsequently seeded in an overlay that was poured over plates containing antibiotics produced by Eh421 (PanA+) and Eh318.

Construction of smaller, antibiotic-encoding clones. DNA from cosmids pCPP702 and pBluescript KS(+) was digested with EcoRI, ligated, and added to competent DH5a cells. White colonies on LB agar amended with ampicillin and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were screened for antibiotic production in a live assay with GA medium or EcMM as the basal medium. The cloned DNA present in colonies that produced zones of inhibition against Ea273 was characterized. A smaller, antibiotic-conferring clone was designated pCPP1051. Similarly, a subclone of pCPP704 was constructed by digesting the cosmid DNA with BamHI and religating. A 32.7-kb BamHI fragment that also included the cosmid vector pCPP9 was designated pCPP719.

Transposon mutagenesis of pCPP1051 and pCPP719. The two subclones, pCPP1051 and pCPP719, were mutagenized with transposons that carried distinct antibiotic resistances (kanamycin and chloramphenicol, respectively) to allow for the construction and selection of a double marker exchange mutant of pantocin A production by DH5a(pCPP1051) while insertion of pantocin B (10) was cloned from pCPP704 into pBR322. This work

<table>
<thead>
<tr>
<th>TABLE 1—Continued</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain, phage, cosmid or plasmid</td>
</tr>
<tr>
<td>pCPP719</td>
</tr>
<tr>
<td>pU1 mini-Tn5Cm</td>
</tr>
<tr>
<td>pCPP722</td>
</tr>
<tr>
<td>pCPP726</td>
</tr>
<tr>
<td>pBR322</td>
</tr>
<tr>
<td>pBR325</td>
</tr>
<tr>
<td>pCPP705</td>
</tr>
<tr>
<td>pCPP810</td>
</tr>
</tbody>
</table>

a Ap′, Cm′, Km′, Na′, Rp′, Sm′, Sp′, Te′, indicate resistance to ampicillin, chloramphenicol, kanamycin, nalidixic acid, rifampin, streptomycin, spectinomycin, and tetracycline, respectively.

b ATCC, American Type Culture Collection; CUPCB; Cornell University Collection of Phytopathogenic Bacteria. Last name denotes isolator or distributor.

RESULTS

Identification and characterization of antibiotic-encoding cosmid libraries. Five of 1,500 members of the genomic library of Eh318 in E. coli JM109 exhibited antibiotic against Ea273 on GA medium. Examination of the EcoRI and HindIII digest patterns of the five cosmids (data not shown) (55) indicated that two of the cosmids, designated pCPP702 and pCPP704, had no bands in common. The restriction enzyme patterns of two of the other cosmids (pCPP701 and pCPP703) appeared similar to those of pCPP702, while the patterns of the fifth cosmid (pCPP705) was distinct from all others. Figure 2 depicts the absence of common bands in pCPP702 and pCPP704 after digesting with EcoRI and a combination of EcoRI and XbaI. This initial genetic difference between these two cosmid clones was supported by detailed genetic mapping of clones, mapping of transposon insertions, and cross-hybridization tests.

Construction of pCPP1051 and pCPP719. Digestion of pCPP702 with EcoRI resulted in seven fragments, whose sizes—11.8, 10.8, 8.4, 4, 3, 2.3, and 1.8 kb (Fig. 2)—total 42.1 kb, a number which includes the cosmids vector pCPP9 (5.3 kb). Recombinant plasmid pCPP1051 consisted of an 11.8- and an 8.4-kb EcoRI fragment from pCPP702 cloned into pBluescript. DH5a(pCPP1051) inhibited Ea273 when grown on EcMM. A restriction enzyme map of the 20.2-kb insert of pCPP1051 was generated (Fig. 3). The genetic region involved in the biosynthesis of pantocin B (10) was cloned from pCPP704 into pCPP719. This clone conferred upon DH5a the ability to inhibit the growth of Ea273. A detailed restriction enzyme map of pCPP719 was generated (Fig. 4).

Transposon mutagenesis and construction of clones for mutagenesis of Eh318. The two subclones, pCPP1051 and pCPP719, were subsequently mutagenized with Tn5 and mini-Tn5Cm, respectively, to generate constructs potentially useful for marker exchange mutagenesis of Eh318 and to determine the approximate locations and sizes of pantocin-encoding regions in the subclones. Colonies of DH5a(pCPP1051::Tn5) and DH5a(pCPP719::mini-Tn5Cm) that had lost the ability to inhibit growth of E. amylovora were selected, and the transposon insertions were mapped. The Tn5 insertion 122 abolished pantocin A production by DH5a(pCPP1051) while insertion 326 did not. (Fig. 3). The 29 mini-Tn5Cm-insertions all abol-
ished pantocin B production by DH5α(pCPP719) (Fig. 4). Insertion A14 was chosen for marker exchange mutagenesis. The 11.8-kb EcoRI fragment of pCPP719 containing insertion 122 was cloned from pCPP745 (a pantocin A-deficient Tn5 mutant of pCPP1051) into pBR325 to generate pCPP726 (Fig. 3). Similarly, a 10.3-kb ClaI fragment of pCPP719 containing insertion A14 was cloned from pCPP810 (the mini-Tn5Cm mutant of pCPP719) into pBR322 to generate pCPP723 (Fig. 4).

**Marker exchange mutagenesis.** Plasmids pCPP726 and pCPP723 were separately introduced into Eh318 and subsequently cured, allowing for homologous recombination of the transposon insertions into the genome of Eh318. A marker exchange mutant originating from Eh318(pCPP726) was designated Eh421 (PanA−), and a mutant originating from Eh318(pCPP723) was designated Eh439 (PanB−). Eh421 (PanA−) was mutagenized using pCPP723 to generate a mutant of Eh318 that carried both transposons, designated Eh440 (PanAB−). Eh440 (PanAB−) was identified by its complete lack of antibiosis toward Ea273 in a live test. The genomic DNA of Eh421 (PanA−) was hybridized to a radioactive probe of a 3.9-kb XbaI-HindIII fragment of pCPP7015 (Fig. 3) and that of Eh439 (PanB−) and Eh440 (PanAB−) to a probe of the 23-kb SmaI fragment of pCPP719 (Fig. 4), respectively. Since Eh440 (PanAB−) was derived from Eh421 (PanA−), it was not necessary to confirm the location of the Tn5 insertion. In all cases, the mutants were true marker exchange mutants, based on analysis of the Southern blots (see details of analysis below) (55). DNAs of Eh421 (PanA−) and Eh318 were digested with NotI and XbaI, HindIII, BglII, and EcoRI and probed with the 3.9-kb insert DNA of pCPP717. The Tn5 insertion was detected in the expected position based on the analysis of several enzyme digests (data not shown) (55). The 12-kb NotI-XbaI fragment of Eh318 was replaced by a 10- and a 3.2-kb fragment in Eh421 (PanA−). The 1.45-kb HindIII fragment of Eh318, which had been mutated by the inserted transposon, was absent in Eh421 (PanA−) as expected.

The analysis of the insertion sites in Eh439 (PanB−) and Eh440 (PanAB−) using a radioactive probe of the 23-kb SmaI fragment of pCPP719 indicated successful marker exchange also of the mini-Tn5Cm insertion. The combined digest with XbaI and SalI showed that the 5-kb XbaI fragment, which contained mini-Tn5Cm, was absent in Eh439 (PanB−) and Eh440 (PanAB−) (data not shown) (55). That fragment had been replaced by two new genomic hybridizing fragments, 2.5 and 6.7 kb in size, through the presence of a SalI site in one end of the transposon. Moreover, in the BamHI and ClaI double digest of Eh439 (PanB−) and Eh440 (PanAB−) DNA, the native 6.6-kb ClaI fragment was absent from the blot as expected (55).
The mutants of Eh318 that were defective in synthesis of one of the antibiotics, i.e., Eh421 (PanA\(^{+}\)) and Eh439 (PanB\(^{+}\)), produced inhibition zones against Ea273 in the chloroform assay that at first glance looked similar in size to or slightly smaller than those produced by Eh318 (data not shown). However, the zones were single in nature, whereas Eh318 produced a double halo (Fig. 1). In the test in which overlays were seeded with Ea273R421 (the variant of Ea273 that was resistant to the PanA\(^{+}\) allele) and Eh318 and Eh421 (PanA\(^{+}\)) were the producers, only the plates in which Eh318 had grown had a zone of inhibition (data not shown).

Cross-hybridization data. EcoRI-XbaI-, EcoRV-, and EcoRI-digested DNA of Eh318, pCPP702, pCPP704, and pCPP1051 was hybridized to a 3.9-kb XbaI-HindIII fragment of pCPP1051 that encompassed the DNA region of the Tn5 insertion site 122 in the pantocin A-deficient clone pCPP726 (Fig. 3). The only hybridizing fragments were those of Eh318, pCPP702 or pCPP1051 origin, as indicated in Fig. 2: a 9.9-kb EcoRI-XbaI (lane 1), a 7.2-kb EcoRI-XbaI (lanes 2 and 4), and a 9.9-kb EcoRV (lane 9), and a 12-kb EcoRI (lane 16 and 18) band (data not shown).

Effect of amino acid supplementation on activity of pantocins. The antibiotic activity of DH5\(\alpha\)(pCPP702) to \(E.\ amylovora\) was inhibited by the presence of histidine but not arginine; however, arginine but not histidine inhibited the activity of DH5\(\alpha\)(pCPP704). However, the zone of inhibition produced by Eh318 in an overlay seeded with \(E.\ amylovora\) was not affected by the presence of either amino acid, when added separately or together.

Effect of amino acid supplementation on activity of pantocins. The antibiotic activity of DH5\(\alpha\)(pCPP702) to \(E.\ amylovora\) was inhibited by the presence of histidine but not arginine; however, arginine but not histidine inhibited the activity of DH5\(\alpha\)(pCPP704). However, the zone of inhibition produced by Eh318 in an overlay seeded with \(E.\ amylovora\) was not affected by the presence of either amino acid, when added separately or together.

Spectrum of activity. The antibacterial spectra of activity of Eh318, DH5\(\alpha\)(pCPP702), Eh439 (PanB\(^{+}\)), DH5\(\alpha\)(pCPP704), Eh421 (PanA\(^{+}\)), and Eh440 (PanAB\(^{+}\)) are summarized in Table 2. The antibacterial spectra of the two antibiotics produced by Eh318 that are inhibitory to \(E.\ amylovora\) differ somewhat but overall are highly similar. Typically, the antibiotics inhibit close relatives of Eh318, such as species of \(P.\ agglomerans\), \(E.\ amylovora\), \(E.\ coli\), \(P.\ aeruginosa\), and \(S.\ marcescens\). Eh318 was resistant to pantocin A but not pantocin B (Fig. 5; Table 2). In addition, we can conclude from the data that a third antibiotic of Eh318 inhibits some nonenterics, judging by the spectrum of activity of Eh440 (PanAB\(^{+}\)), and this antibiotic does not inhibit \(E.\ amylovora\).

DISCUSSION

\(P.\ agglomerans\) strain Eh318 produces two antibiotics that are active against \(E.\ amylovora\) based on genetic, biological, and chemical evidence. We have proposed to name them pantocin A, whose biosynthetic genes are present in pCPP702 and pCPP1051, and pantocin B (10), whose biosynthetic genes are present in pCPP704 and pCPP719. The DNA regions responsible for the synthesis of pantocin A and B are distinct based on size, restriction maps (Fig. 3 and 4), and lack of hybridization of DNA for biosynthesis of pantocin A to that for biosynthesis of pantocin B (Fig. 2). The activities of the two antibiotics also are clearly distinct. The activity of DH5\(\alpha\)(pCPP702) is lost in the presence of histidine, while that of DH5\(\alpha\)(pCPP704) is lost in the presence of arginine. The spectra for the antibiotics produced by strains that synthesize one or both antibiotics are distinct (Table 2). The double zone of inhibition produced by Eh318 in an overlay seeded with Ea273 (Fig. 1) likely is due to the presence of two antibiotics, of which one diffuses further than the other. Mutants that produce only one of the pantocins, i.e., Eh421 (PanA\(^{+}\)) and Eh439 (PanB\(^{+}\)) produce single, discrete zones in overlays of Ea273. A variant of Eh273 with spontaneous resistance to the antibiotic produced by Eh421 (PanA\(^{+}\)), i.e., to pantocin B is sensitive to Eh318.

Chemical data also suggest that the two antibiotics are distinct. Pantocin B was recently identified as (R)-N-[(\(S\))-2-amino-2-propanoylamino]-methyl]-2-methanesulfonyleuccamine acid, a peptide of 296 Da (10). It is sufficiently stable to allow for its isolation and characterization from culture supernatants of DH5\(\alpha\)(pCPP719). In contrast, pantocin A is labile to extremes of pH, and therefore it has been recalcitrant to isolation and structural characterization using similar procedures as employed for pantocin B (M. Jin, personal communication).
TABLE 2. Inhibition of bacteria by Eh318 and derivatives that produce one or two pantocins that inhibit E. amylovora.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Inhibition by&lt;br&gt; Dh5α (Pan AB&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>Dh5α (pCPP1051) (Pan AB&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>Eh349 (Pan A&lt;sup&gt;b&lt;/sup&gt;)</th>
<th>Dh5α (pCPP704) (Pan B&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>Eh421 (Pan B&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>Eh440 (Pan AB&lt;sup&gt;a&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. amylovora Ea273</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Pantoea sp. Eh112Y</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>P. agglomerans Eh252</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>P. stewartii 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>--</td>
</tr>
<tr>
<td>E. carotovora subsp. carotovora B12</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>NT</td>
</tr>
<tr>
<td>E. carotovora subsp. betavasculorum 101</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
<td>(+)</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>E. chrysanthemi 3665</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E. aerogenes 1422/77</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>S. marcescens Ser 101</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>K. pneumonia</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>P. putida W713</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>P. syringae pv. tomato DC 3000</td>
<td>+</td>
<td>--</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>X. campestris pv. pelargonii Xpel-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A. tumefaciens C58</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

<sup>a</sup> Combined results of a chloroform assay for E. coli strains carrying cosmids and Eh318 and a live assay for marker exchange mutants and Eh318, using GA base medium and GA overlay.

<sup>b</sup> Symbols: +, clear inhibition zone; (+), diffuse inhibition zone; --, no inhibition zone.

<sup>NT</sup>, not tested.

<sup>d</sup>V, variable.

Subcloning and transposon mutagenesis data suggest that the genetic region involved in the biosynthesis of pantocin A is at least 7.5 kb, while that of pantocin B is at least 18.5 kb. The Tn5 insertion 326 at map position 4.3 (Fig. 3) does not abolish the antibiotic activity of Dh5α (pCPP1051). A subclone of pCPP1051 that carries only the 11.8-kb EcoRI fragment confers pantocin A production on Dh5α (55). Hence, the genes for pantocin A biosynthesis lie within a 7.5-kb region. Based on the mapping of 29 mini-Tn5 insertions in pCPP19 (Fig. 4), the biosynthetic region for pantocin B is at least 18.5 kb. The genetic regions involved in the synthesis of antibiotics of other strains of P. agglomerans-P. dispersa are under investigation elsewhere. For Eh1087, a New Zealand strain, a 2.2-kb region was found to be essential (31). In Eh252, another New York strain, deletion and complementation analysis of transposon-bearing clones delimited the mccEh252 biosynthetic genes to a 2.4-kb region (44). In C9-1, a Michigan strain, a cosmide clone, AA818, was identified from a genomic DNA library that confers on Dh5α the ability to synthesize herbacolin O (11).

Pantocin A and pantocin B have similar but distinct spectra of activity. However, only pantocin B inhibited P. agglomerans Eh252, Xanthomonas campestris pv. pelargonii and E. coli DH5α (Table 2). The two pantocins produced by Dh5α carrying the cosmids mainly inhibited enteric strains of bacteria. They are together solely responsible for the inhibition of Erwinia stewartii, Erwinia chrysanthemi, Erwinia carotovora subsp. carotovora, and E. amylovora by Eh318, judging by the absence of inhibition of these strains by Eh440 (PanAB<sup>a</sup>) (55). This result is consistent with those of El-Goorani and coworkers, who found that the antimicrobial activity of Eh318 primarily affects enterics, with the exception of Rhodococcus fascians (15), and the same was true for several other strains of Pantoea spp. (15, 25, 28). The antibiotics of several Pantoea strains were initially designated bacteriocins due to their inhibition primarily of closely related species (3). Based on the inhibition by Eh440 (PanAB<sup>a</sup>), the Erwinia strain of Streptococcus faecalis, X. campestris pv. pelargonii, Pseudomonas syringae pv. tomato, and Klebsiella pneumoniae, Eh318 likely produces a third antibiotic compound that is ineffective on erwiniats.

Earlier studies have found that the zone(s) of inhibition produced by Eh318 in an overlay seeded with E. amylovora is abolished or reduced in diameter to 50% or less in the presence of a combination of histidine and arginine (50). However, in our experiments, using as much as 10 mg of the two amino acids per ml in the overlay did not prevent the formation of zones. Perhaps numbers of Eh318 cells used or the time they were allowed to produce antibiotic overcame the arginine-histidine supplementation effect. The arginine effect on pantocin B activity likely is due to the redundancy of the arginine biosynthetic pathway, the target of pantocin B (10), when arginine is supplied exogenously.

Pantocin A is inactive in the presence of histidine, which is a characteristic of most antibiotics produced by Pantoea species that have been tested (14, 50, 51). MccEh252, the antibiotic produced by P. agglomerans strain Eh252 (J. L. Vanneste, J. Yu, D. C. Cornish, and M. D. Voyle, 7th Int. Congr. Plant Pathol, paper 3.5.4, 1998 [www.bspp.org.uk/icpp98/abstracts].
Bacillus megaterium 
cescens (11, 45, 55), whereas the biosynthetic genes for several other 
sponsible for their synthesis do not reside on native plasmids 
of structurally related compounds. Interestingly, the genes re-
Pantocin A, mccEh252, and herbicolin O may fall into a family 
pantocin B are two distinct compounds, which are produced by 
to the genetic difference, clearly indicate that pantocin A and 
to identify and isolate clones corresponding to distinct biosyn-
and Barbara Sneath for discussing the experiments and techniques, 
and Raymond Fernalld for technical assistance. We thank David Bauer 
exists some structural information is herbicolin O, a 
subsp. are plasmid borne (19, 30, 42).

We have demonstrated the value of using a genomic library 
to identify and isolate clones corresponding to distinct biosyn-
their interactions with 
control of fire blight on apple. Acta Hortic.

ANTIBIOTICS PRODUCED BY 
PANTOEA AGGLOMERANS

tocols for electrophoretic and capillary transfer of DNA and RNA, DNA and 

Erwinia amylovora: techniques, tools and their application. Cornell Univer-
sity, Ithaca, N.Y.

bacteriocin-like substances. Phytopathology 70:459. (Abstract.)


Erwinia herbicola and their interactions with Erwinia amylovora in immature 
pear fruits. Phytopathology 81:121. (Abstract.)

15. El-Goorani, M. A., F. M. Hassanein, and A. A. Shoeb. 1992. Antibacterial and antifungal spectra of antibiotics produced by different strains of 


resistant transposon Tn5 to Erwinia herbicola and the induction of insertion 


24. Hanahan, D., J. Jesse, and D. F. Bloom. 1995. Techniques for transforma-


produced by Erwinia herbicola on Erwinia amylovora. Phytopathology 70:463.


Nucleic Acids Res. 9:2899–2908.


production by Erwinia herbicola. Phytopathology 78:746–750.

...
honey bee-dispersed Erwinia amylovora in pear blossoms and on fire blight control. Phytopathology 83:995–1002.


