Cloning and Transfer of Genes for Antifungal Compounds from *Erwinia herbicola* to *Escherichia coli*

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Received 9 December 1991. Revised 5 March 1993. Accepted 15 March 1993.

Erwinia herbicola **CHS1065 produces antifungal compounds highly related to herbicolin A. In 11 Tn*5* mutants that have lost the antifungal activity, the transposon insertion is located on a 170-kb plasmid present in CHS1065. This plasmid was designated pHER1065. When analyzing these antifungal mutants, it was found that the genes for the biosynthesis of the antifungal compounds were organized in at least two clusters on pHER1065. Upon insertion of the *aph*II gene of Tn*5* and genes for plasmid mobilization in pHER1065, the plasmid could be stably introduced into *Escherichia coli*. All the *E. coli* exconjugants expressed an antifungal activity that was quantitatively and qualitatively comparable to the activity produced by *E. herbicola* CHS1065. Amino acid analysis and molecular weight determinations of the antifungal compound produced by CHS1065 were identical to those of herbicolin A.**

Bacterial characters important for the colonization of specific niches are often plasmid linked. Oncogenicity and opine catabolism genes, which allow the genetic colonization of plant cells by *Agrobacterium tumefaciens*, are located on Ti-plasmids (Caplan *et al.* 1983). Genes involved in the nodulation and nitrogen fixation that allow *Rhizobium* to induce N_2-fixing nodules on leguminous plants are also located on plasmids (Coplin 1982). Also, genes involved in pathogenicity of *Pseudomonas savastanoi, P. phaseolicola*, and *Erwinia stewartii* (Coplin 1982) are plasmid linked. Additionally, in *E. herbicola* it has been shown that biosynthesis of the carotenoid pigment and thiamine prototrophy are located on a large plasmid (Gantotti and Beer 1982). A number of soil microorganisms have been shown to produce secondary metabolites that inhibit the growth of soilborne fungi. Based on these data, a number of organisms have been tested as biocontrol agents for soilborne plant pathogenic fungi (Cook and Baker 1983). Since the chemical structure of these secondary metabolites is complex, the biosynthetic pathways of these compounds are expected to be analogous in genetic complexity to the ones of peptide antibiotics (Katz and Demain 1977).

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MPMI, Vol. 6, No. 4, pp. 474-480

E. herbicola is also known to produce specific metabolites like a carotenoid pigment suggested as a protectant from ultraviolet light (Bennetzen *et al.* 1988), ice nucleation-active protein (Lindow *et al.* 1978), and amino-acid-containing compounds with antifungal activity (Wood 1966).

E. herbicola strains also have phenotypes that make them competitive with other microorganisms in different niches. Some *E. herbicola* strains have proven to be antagonistic to *E. amylovora*, the causal agent of fire blight on pear and apple trees (Chatterjee and Gibbins 1969). Other strains produce peptides with antifungal activities (Wood 1966). Herbicolins are depsi-glycopeptides containing L- and D-amino acids. The β-hydroxymyristoyl group has been shown to be a natural component of lipid A of bacterial cell membranes (Kusumoto *et al.* 1985). One herbicolin-producing strain has also been used as a biocontrol agent of *Fusarium* on wheat (Kempf and Wolf 1989).

In this work, evidence is presented that the strain *E. herbicola* CHS1065 (Van Outryve *et al.* 1988) produces an antifungal compound highly similar to herbicolin A. Genes for the production of this antifungal compound are localized on a plasmid. When the plasmid of CHS1065 was transferred to *Escherichia coli*, this new host produced antifungal compounds in comparable amounts to CHS1065.

RESULTS

Biological activity of CHS1065.

CHS1065 was obtained during an isolation of bacteria from chicory witloof seeds (*Cichorium intybus* L. var. *foliosum* Hegi) (Van Outryve *et al.* 1988). The strain was identified as *Erwinia herbicola* by the API 20E system (API, Montailieu Vercieu, France). When CHS1065 was tested for the ability to inhibit the growth of fungi, all 27 different plant pathogenic fungi tested were inhibited (Table 1).

Characterization of the antifungal compound of CHS1065.

Amino acid analysis, performed on the purified antifungal compound from CHS1065, showed that this compound had the same amino acid composition as herbicolin A (Aydin *et al.* 1985). A fast atom bombardment (FAB)-mass spectroscopic analysis confirmed a molecular weight of 1,302, which is identical to that of herbicolin A. When a second high-performance liquid chromatography (HPLC) fraction with antifungal activity was analyzed for amino

acids, it was found to be identical to herbicolin A, suggesting that CHS1065 produces two structurally related antifungal compounds.

Transposon mutagenesis of CHS1065 and localization of the mutated site.

When 1,230 kanamycin-resistant (Km^r) exconjugants were screened for their antifungal activity against *C. lindemuthianum,* 11 mutants were found and tested for antifungal activity against 10 different fungi. None of the mutants could inhibit any of the tested fungi. They were designated CHS1065-1 to CHS1065-11.

Plasmid analysis by the method of Kado and Liu (1981) revealed that CHS1065 harbored a plasmid of approximately 170 kb. This plasmid will hereafter be referred to as pHER1065. All nonantifungal mutants had a plasmid of the size of pHER1065. An agarose gel with undigested plasmid and chromosomal DNA from CHS1065 wild type and the nonantifungal mutants was blotted and hybridized with the *aph*II gene of Tn*5*, isolated from pKC7. As can be seen in Figure 1, a hybridization signal occurred in all 11 mutants at a site corresponding to the 170-kb plasmid. Chromosomal DNA did not hybridize in any of the lanes. By using the same probe on digested total DNA, it was shown that the mutants carried one insertion of Tn*5* per mutant (*data not shown*).

Antifungal activity of mutant CHS1065-9.

To confirm that the biosynthesis of herbicolin is inactivated in the mutants, the culture supernatant from one mutant (CHS1065-9) and from wild-type CHS1065 was fractionated on Bond Elut columns and HPLC (see Materials and Methods). The results shown in Figure 2 indicate that the production of antifungal compounds found in CHS1065 is completely abolished in mutant CHS1065-9. This also means that by the inactivation of one gene, as in this mutant, the production of both antifungal compounds is inhibited, suggesting that these antifungal compounds have a common biosynthetic pathway or a common regulatory gene.

The abbreviation *hin* is proposed for the genes involved in the herbicolin production. The nonantifungal mutants of CHS1065 will subsequently be designated as Hin^- mutants.

Screening for herbicolin-producing cosmid clones.

In order to analyze these genes in more detail, it was decided to clone the genes for herbicolin from the pHER1065 plasmid. This was done in cosmid pMOPI (Brady *et al.* 1984; Tuite 1969). We screened 950 *E. coli* cosmid clones for the expression and secretion of antifungal compounds with *C. lindemuthianum* spore plates. However, no clone was found that inhibited the fungus, although 95% of the cosmid clones harbored inserts with an average size of 22 kb. Yellow-pigmented *E. coli* clones appeared in a frequency of 1/50 of the clones. They most likely obtain their pigment from cosmids containing the genes for the biosynthesis of the yellow pigment of CHS1065. The genes conferring this yellow pigmentation are known to be

Table 1. Fungi inhibited by *Erwinia herbicola* CHS1065

Aphanomyces leavis PGSF225
Aspergillus niger PGSF163
Beauveria bassiana PGSF150
Botrytis spp. PGSF29
B. cinerea PGSF100
Cladosporium herbarum PGSF136
Colletotrichum lindemuthianum PGSF58
Coniella diplodiella PGSF123
Fusarium moniliforme PGSF96
F. nivale PGSF56
F. roseum PGSF15
Gaeumannomyces graminis var. *tritici* PGSF65
Penicillium chrysogenum PGSF1
P. funiculosum PGSF162
Phoma betae PGSF79
P. exigua PGSF94
Pyricularia oryzae PGSF207
Pythium ultimum PGSF185
Rhizoctonia cerealis PGSF23
R. solani PGSF18
Rhizopus oryzae PGSF176
Saprolegnia parasitica PGSF186
Sclerotinia minor PGSF87
S. sclerotiorum PGSF55
Thielaviopsis basicola PGSF48
Tolaeemyces reachyspermius PGSF222
Verticillium inventa PGSF140

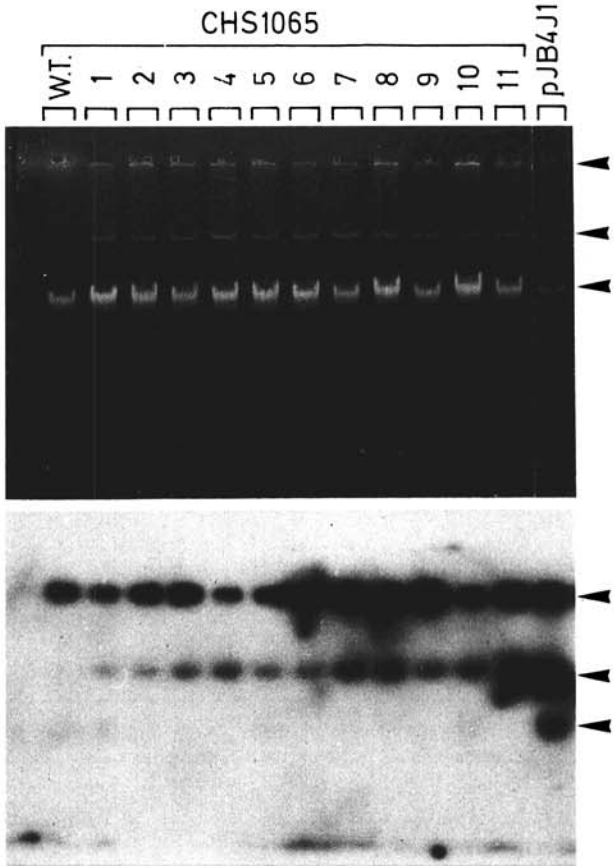

Fig. 1. Hybridization of the Km^r of Tn*5* to CHS1065 and the Hin^- mutants. Plasmid DNA was isolated from CHS1065 and the 11 Hin^- mutants. This DNA was separated in an agarose gel. The gel was blotted and hybridized to the *aph*II gene isolated from pKC7. pJB4JI contains Tn*5* and was used as a positive control in the hybridization experiment. The lower panel shows the autoradiographic film. The arrows indicate, from the top, the slots, the position of pHER1065, and the position of the chromosomal DNA.

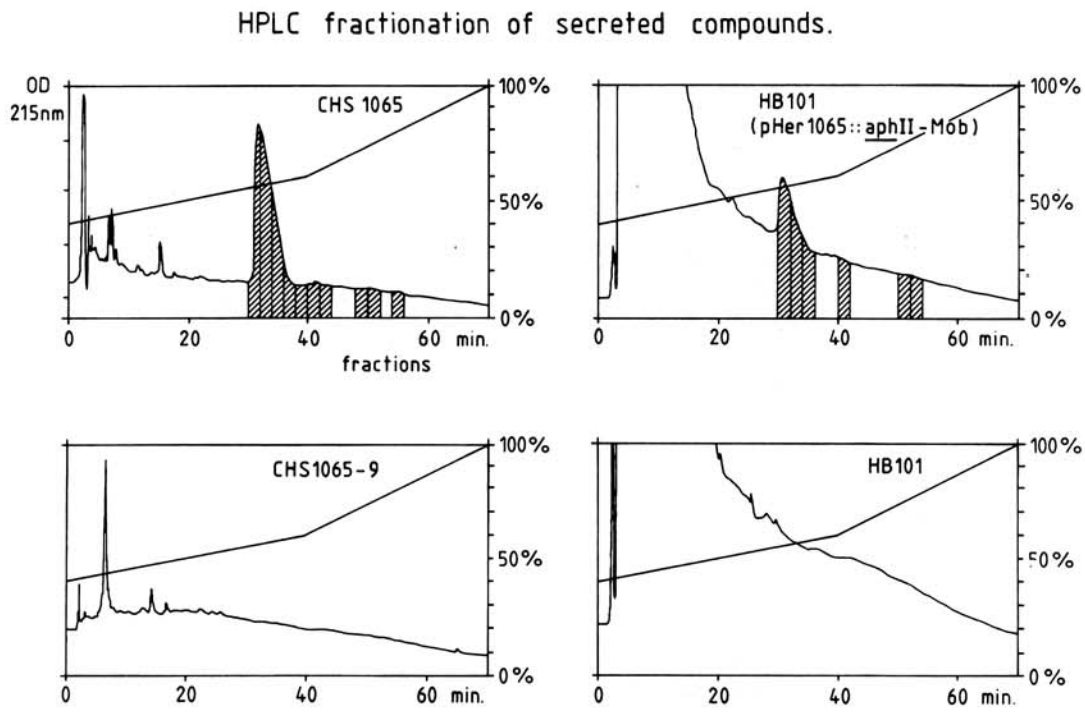

Fig. 2. High-performance liquid chromatogram of culture supernatants of CHS1065, CHS1065-9, HB101, and HB101(pHER1065::*aph*II-Mob). The antifungal fractions are indicated by filled-in peaks. The compounds were eluted with an acetonitrile/water gradient as described in Materials and Methods.

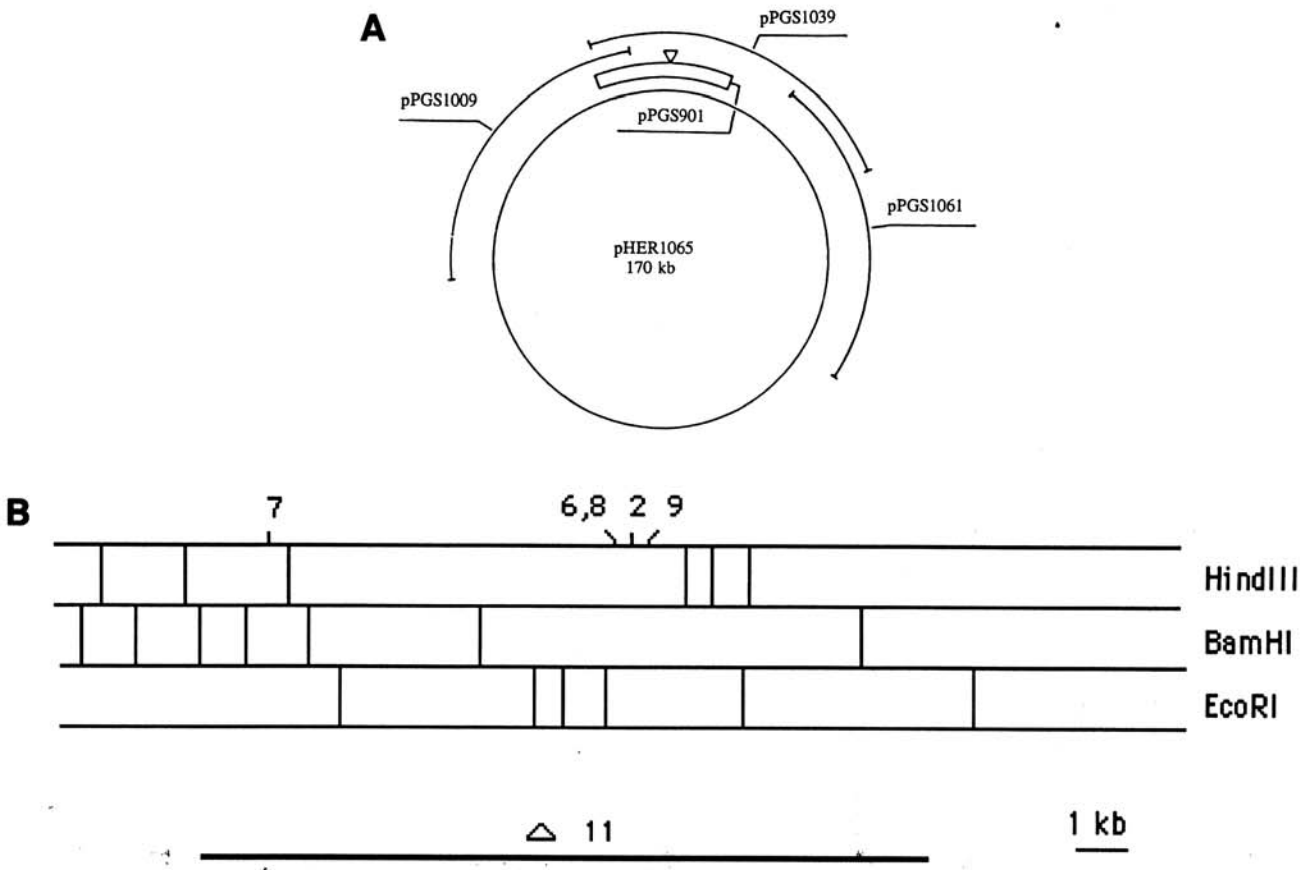

Fig. 3. A, A schematic map indicating the sequence covered by the cosmids used to localize the Tn*5* insertions. **B,** Localization of the Tn*5* insertions on pHER1065, on the basis of hybridization of pPGS1009 and pPGS1039 to total DNA of the 11 mutants and wild-type CHS1065.

plasmid linked in *E. herbicola* and organized in at least two operons covering 12.4 kb (Roeder and Collmer 1985). This was taken as an estimate for the cloning efficiency of intact large segments of the plasmid. Moreover, they are an example of a gene cluster from this organism that is properly expressed in *E. coli*.

Localization of Tn*5* in the *Hin*$^-$ mutants.

To localize the site of mutation in the different Tn*5* induced *Hin*$^-$ mutants, cosmids pPGS901, pPGS1009, pPGS1039, and pPGS1061—covering 65% of pHER1065 (Fig. 3A)—were hybridized to total DNA of the 11 mutants and wild-type CHS1065. Total DNA was isolated by the method of Dhaese *et al.* (1979). The results of these hybridizations are schematized in Figure 3B. Mutants CHS1065-2, -6, -8, and -9 carry Tn*5* insertions in a 7.3-kb *Hin*dIII fragment. The distance between the insertion sites is 200–300 bp. Tn*5* in mutants CHS1065-6 and -8 are probably located at the same point. Mutant CHS1065-7 is mutated in a 2.3-kb *Bam*HI fragment. CHS1065-11 has suffered a deletion that removes more than 20 kb in this region. The exact position of Tn*5* in the other mutants was not determined, since they are positioned outside the sequence covered by the three cosmids, indicating that they are located at least 39 kb from the 7.1-kb region. It was concluded that the genes responsible for the production of herbicolin in CHS1065 are separated in at least two regions. In one 7.1-kb region there are at least two genes responsible for the production of antifungal compounds.

Construction of pHER1065::*aph*II-Mob and mobilization of this plasmid to *E. coli*.

By insertion of an antibiotic marker and mobilization functions onto pHER1065 it was be possible to select for the mobilization of this plasmid to another host.

pSUP5011 (Trieu-Cuot and Courvalin 1983) contains the mobilization functions of RP4 cloned in the single *Bam*HI-site of Tn*5*. The internal *Hin*dIII fragment from Tn*5* on pSUP5011 contains both the *aph*II gene for Tn*5* and the Mob functions from the broad-host range plasmid RP4. This *Hin*dIII fragment was cloned in the unique *Hin*dIII site of pPGS2100 which contains a randomly isolated *Bam*HI fragment of pHER1065 in pPGS2000. pPGS2200 was mobilized to CHS1065R at a frequency of 3×10^{-4} per acceptor, using pRK2073 as a donor plasmid. In CHS1065R a double recombination event was selected for by isolating Km^r Ap^s colonies. The loss of ampicillin resistance is based on the instability of pBR-derived replicons in medium containing low concentrations of phosphate (Simon 1984). A culture of CHS1065R (pPGS2200) was grown for 32 hr at 28° C in minimal medium containing 1 mM phosphate. When the culture was diluted and plated on kanamycin and ampicillin, Ap^r colonies appeared with a frequency of 4×10^{-7} per Km^r colony. This showed that the low-phosphate treatment efficiently selects against pPGS2200, and that in this way a marker exchange mutagenesis was obtained. Six Km^{r}Ap^s colonies were tested for their antifungal activity and were all found to be positive. The marking of pHER1065 with *aph*II and Mob was confirmed with a Southern blot analysis using pSUP5011 as a probe. RP4-4 was used as donor plasmid in a mobilization of pHER1065::*aph*II-Mob from CHS1065R to HB101. Km^r HB101 exconjugants were obtained with a frequency of 2×10^{-3} per acceptor. Six of these exconjugants were tested for antifungal activity against *C. lindemuthianum*, and they were all found to be positive (Fig. 4). All six strains carried a plasmid of the same size as pHER1065.

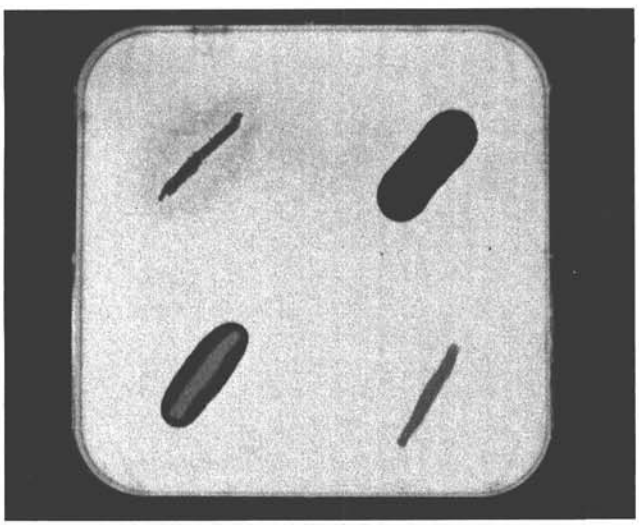

Fig. 4. Antifungal activity of CHS1065-9, CHS1065, HB101, and HB101(pHER1065::*aph*II-Mob) going clockwise from the upper left corner. The strains were tested on Luria broth with *Colletotrichum lindemuthianum* PGSF58 as the test fungus.

Analysis of the antifungal compound produced by HB101(pHER1065::*aph*II-Mob).

The antifungal compound produced by HB101(pHER1065::*aph*II-Mob) was isolated and shown to have an amino acid composition identical to the compound produced by CHS1065. The antifungal compound produced by the *E. coli* clone had a retention time identical to the antifungal compound produced by CHS1065 in a coinjection HPLC analysis (Fig. 2) and also had an identical molecular weight as determined by FAB-mass spectroscopy.

Hybridization of pPGS1009 and pPGS1039 to nonantifungal *Erwinia* strains.

In the screening where CHS1065 was isolated, a number of nonantifungal *E. herbicola* strains were also isolated. All these strains contained plasmids with a size of about 150–200 kb (*data not shown*). To determine whether these isolates carry the genes for the biosynthesis of the antifungal compound but are impaired in another step in the production, or if a genetic rearrangement in the genes responsible for the antifungal compounds could be used to explain the lack of the antifungal compound, a hybridization experiment was performed with the isolated cosmid clones as probes. Total DNA was prepared from 7 nonantifungal *E. herbicola* strains. pPGS1009 and pPGS1039 were used as probes to digested total DNA from these strains in a Southern blot analysis. As shown in Figure 5, major differences appear between pHER1065 and the other *Erwinia* strains. In the sequence covered by pPGS1039, one or more bands was missing in the nonantifungal strains,

whereas in the region covered by pPGS1009, large rearrangements had taken place. The rearrangements were of different sizes, suggesting that the nonantifungal *Erwinia* strains had suffered deletions rather than that CHS1065 had acquired an insertion of novel sequences. Large differences could also be seen between the different nonantifungal strains, indicating that these plasmids are not stable and that genetic rearrangements readily occur.

DISCUSSION

Indications for the involvement of pHER1065 in the production of an antifungal compound were obtained when 11 different nonantifungal transposon mutants were found to be mutated on the plasmid. A second antifungal compound produced by an *Erwinia* strain, herbicolin B, was reported by Aydin *et al.* (1985). This compound is structurally related to herbicolin A and elutes later during the HPLC fractionation. This might indicate that the second antifungal compound produced by CHS1065 is herbicolin B.

The fact that *E. coli* carrying pHER1065::*aph*II-Mob inhibits fungi suggests that all the genes necessary for the biosynthesis of herbicolin are present on pHER1065. If the depsi-peptide ring of herbicolin without the fatty acid chain has no antifungal activity, one could speculate that the circular peptide is synthesized by genes on the plasmid pHER1065, and that the hydroxymyristic acid is connected to the peptide ring while attached to the cell membrane, and in this way forms the active herbicolin. This binding might then result in the release of the antifungal compound. However, we have no experimental proof to support this speculation.

The isolation and purification of herbicolin described here was done in two steps. Fractionation on a solid-phase extraction column (Bond Elut) and a subsequent HPLC purification. This purification scheme is less elaborate than the Servachrom column separation, counter-current distribution, followed by a Sephadex column separation as described by Wood (1966).

The results reported above suggest that the antifungal compound produced by CHS1065 could be used as a control agent for agriculturally important fungal diseases. This could be achieved by introducing the herbicolin-producing plasmid into a root-colonizing bacterium, and thereby producing herbicolin along the roots of the plants.

MATERIALS AND METHODS

Media.

Media used in this work were potato-dextrose agar (PDA) and potato-dextrose broth (Winkelmann *et al.* 1980), minimal medium A (Miller 1972), and Luria broth (Maniatis *et al.* 1982).

Strains.

E. coli strains used were HB101 (Boyer and Roulland-Soussoix 1969), and K51R, Rif^r (rifampicin derivative of K504; Van Outryve *et al.* 1988). *E. herbicola* strains were CHS1065 antifungal, pigmented; CHZ17 antifungal, pigmented; CHS1102 nonantifungal, pigmented; CKS1095 nonantifungal, pigmented; CHS1087 nonantifungal, pigmented; CHS1099 nonantifungal, pigmented; CHS1092 nonantifungal, pigmented; CHS1031 nonantifungal, pigmented; CHS1104 nonantifungal, nonpigmented (Van

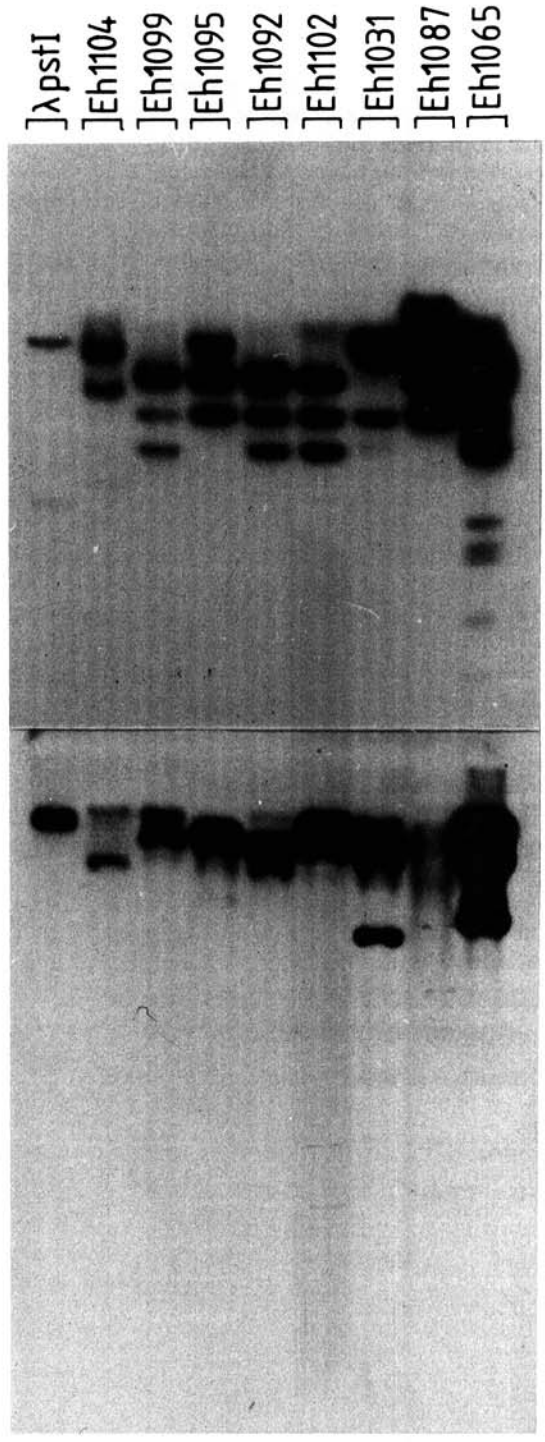

Fig. 5. Hybridization of pPGS1009 and pPGS1039 to total DNA of *Erwinia herbicola* CHS1065 and nonantifungal *E. herbicola* strains isolated from chicory witloof (*Cichorium intybus* L. var. *foliosum* Hegi). Total DNA was isolated from *E. herbicola* CHS1104, CHS1099, CHS1095, CHS1092, CHS1102, CHS1031, CHS1087, and CHS1065. The upper panel shows the results of the hybridization using pPGS1039 as probe; pPGS1009 was used as probe in the lower panel.

Outryve *et al.* 1988); and CHS1065R, Rif^r derivative of CHS1065.

Rifampicin resistant derivatives were isolated by plating 100 μl of a saturated culture on medium containing 100 μg/ml of rifampicin.

Antibiotics.

Antibiotics were used in the following concentrations: ampicillin (Ap) 100 μg/ml, kanamycin (Km) 25 μg/ml, rifampicin (Rif) 100 μg/ml, tetracycline (Tc) 10 μg/ml, chloramphenicol (Cm) 25 μg/ml, and nalidixid acid (Nal) 10 μg/ml.

Assay for antifungal activity.

To test fungi for inhibition by CHS1065, a petri dish assay was used. In this assay, an agar plug taken from a plate covered with fungal spores was put in the center of a plate of PDA, and the bacterium was placed at the edge of the plate. This plate was then incubated at 22° C for 2–6 days. To test fractions from the different purification steps, spore plates of *Colletotrichum lindemuthianum* and *Penicillium chrysogenum* were used. *C. lindemuthianum* was grown for 7 days on PDA at 24° C. The spores were washed off with 10 mM $MgSO_4$ containing 15% glycerol. The concentration of the spore solution was determined in a Petroff-Hausser counting chamber. Aliquots (500 μl) of 10^7 spores/ml were frozen at −80° C. A 100-μl spore sample was added to 20 ml of medium (cooled to 45° C) and poured into a 9-cm diameter petri dish. Wells were made with a cork borer into which 50 μl of the solution to be tested was pipetted. Spore plates of *P. chrysogenum* were made in an identical way.

Large-scale preparation of plasmid pHER1065 of CHS1065.

The method used for the isolation of pHER1065 was described by Hirsch *et al.* (1980) for the isolation of the Ti-plasmid from *Agrobacterium tumefaciens*.

Transposon mutagenesis.

A *Pseudomonas aeruginosa* plasmid, pM075, was used as a transposon donor. This plasmid is a derivative of R91-5 carrying Tn*5*. R91-5 is a *Pseudomonas* plasmid with a narrow host range of replication; however, it conjugates to a broad range of bacteria (Chandler and Krishnapillai 1977). When *P. aeruginosa* (pM075) was conjugated with CHS1065R, Km^r exconjugants were obtained with a frequency of 1×10^{-6} per acceptor. Km^r mutants of CHS1065 appeared spontaneously with a frequency of 1×10^{-8}. pM075 could not be detected in any of 12 randomly picked Km^r exconjugants when analyzed by the method of Kado and Liu (1981). Auxotrophic mutants were found with a frequency of 0.9%.

Construction of pGS2000.

pBR325 was cut with *Eco*RI and *Hin*dIII. After a filling-in reaction, the mixture was diluted to 0.4 μg/ml for a blunt-end ligation overnight. The ligation mixture was used to transform HB101, and transformants were selected on ampicillin. Cm^s colonies were screened for by transferring the Ap^r colonies to chloramphenicol plates. The deletion of this *Eco*RI–*Hin*dIII fragment did not abolish the tetracycline resistance.

Isolation and purification of the antifungal compound.

Different media were used for the production of antifungal compounds from *Erwinia* and *E. coli*. For CHS1065 potato-dextrose broth was used, and *E. coli* was grown in Luria broth. Cultures (500 ml) in 2-L Erlenmeyer flasks were grown for 4 days at 22° C in a shaker (model G25, New Brunswick Scientific, Edison, NJ) set at 150 rpm. The cells were sedimented by centrifugation at 5,000 rpm for 15 min in a centrifuge (model RC-3B, Sorvall), and the cellfree culture supernatant was passed through a reversed phase cartridge (about 200 ml/cartridge; Bond Elut C8, Analytichem, Harbor City, CA). Elution of the extracted compounds was achieved with acetonitrile in water (10–100% acetonitrile in 10% steps). After drying and dissolving the fractions in water/acetone (50:50 mixture), their antifungal activity was assayed for by spotting 1/30 of each fraction on a *P. chrysogenum* spore plate. The active fractions were purified further on an HPLC system (600 multisolvent delivery system: column, ALLTECH-RSL, 25 cm × 4.6 mm i.d., packed with 5 μm RoSil C8; 990 photodiode array detector; Waters Chromatography Div., Milford, MA) using a gradient of acetonitrile in water containing 0.02% trifluoroacetic acid as eluent (0–40 min, 40–60% MeCN; 40–70 min, 60–100% MeCN; flow, 1 ml/min; retention time, 31.7 min; yield, 1 mg/100 ml supernatant).

Amino acid analysis.

The amino acid composition was determined with an amino acid analyzer (Biocal) after total hydrolysis in 6 M HCl. The phenylthiohydantoin (PTH)-amino acids were identified using a Waters HPLC system equipped with a cyanopropyl analytical column (IBM Instruments, Norwich, U.K.), using the gradient elution system described by Hunkapiller *et al.* (1983).

FAB-mass spectroscopic analysis.

The peptide were analyzed with a double-focusing mass spectrometer (model M902, AEI) equipped with an INCOS data system. The FAB ion source was equipped with an M Scan atom gun. Methanol solutions of the peptides (2 μl containing about 1–10 μg) were dropped in glycerol on the stainless steel target of a direct insertion probe. That solution was bombarded with 9.5 kV argon atoms. Ions were accelerated from the source region at 4 kV and at a total scan time of 20 sec. The spectra were calibrated against a standard of tris(perfluoroheptyl)-*s*-triazine (Ventron).

ACKNOWLEDGMENTS

C. Ampe is thanked for the amino acid analysis, E. Messens for the FAB mass analysis, and S. van Gijsegem for making the figures. P. Tenning was financially supported by Hilleshög AB, Sweden.

LITERATURE CITED

Aydin, M., Lucht, N., König, W. A., Lupp, R., Jung, G., and Winkelmann, G. 1985. Structure elucidation of the peptide anti-

biotic herbicolin A and B. Liebigs Ann. Chem. 41:2285-2300.
Bennetzen, J. L., San Miguel, P. J., Larson, R. A., and Tuveson, R. W. 1988. Structure and protective role of the caretenoid synthesis genes of *Erwinia stewartii*. J. Cell Biochem. Suppl. 12C:246.
Boyer, H. W., and Roulland-Doussoix, D. 1969. A complementation analysis of the restriction and modification of DNA in *E. coli*. J. Mol. Biol. 41:121-136.
Brady, G., Jantzen, N. M., Bernard, H. U., Brown, R., Schutz, G. and Hashimotoh-Gotoh, T. 1984. New cosmid vectors developed for eucaryotic DNA cloning. Gene 27:223-232.
Caplan, A., Herrera-Estrella, L., Inzé, D., Van Haute, E., Van Montagu, M., Schell, J., and Zambryski, P. 1983. Introduction of genetic material into plant cells. Science 222:815-821.
Chandler, P. M., and Krishnapillai, V. 1977. Characterization of *Pseudomonas aeruginosa* derepressed R-plasmids. J. Bacteriol. 130:596-603.
Chatterjee, A. K., and Gibbins, L. M. 1969. Metabolism of phloridzin by *Erwinia herbicola*: Nature of the degradation products, and the purification and properties of phloretin hydrolase. J. Bacteriol. 100:594-600.
Cook, R. J., and Baker, K. F. 1983. The Nature and Practice of Biological Control of Plant Pathogens. American Phytopathological Society, St. Paul, MN.
Coplin, D. L. 1982. Plasmids in plant pathogenic bacteria. Pages 255-281 in: Phytopathogenic Prokaryotes, vol. 2. M. S. Mount and G. H. Lacy, eds. Academic Press, New York.
Dhaese, P., De Greve, N., Decraemer, N., Schell, J., and Van Montagu, M. 1979. Rapid mapping of transposon insertion and deletion mutations in the large Ti-plasmids of Aarobacterium tumefaciens. Nucleic Acids Res. 7: 1837-1849.
Gantotti, B. V., and Beer, S. V. 1982. Plasmid-born determinants of pigmentation and thiamine prototrophy in *Erwinia herbicola*. J. Bacteriol. 151:1627-1629.
Hirsch, P. R., Van Montagu, M., Johnston, A. W. B., Brewin, N. J., and Schell, J. 1980. Physical identification of bacteriocinogenic, nodulation and other plasmids in strains of *Rhizobium leguminosarum*. J. Gen. Microbiol. 120:403-412.
Hunkapiller, M. W., Hewick, R. M., Dreyer, W. J., and Hood, L. E. 1983. High sensitivity sequencing with a gas-phase sequenator. Methods Enzymol. 91:399-412.
Kado, C. I., and Liu, S.-T. 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365-1373.
Katz, E., and Demain, A. L. 1977. The peptide antibiotics of *Bacillus*: Chemistry, biogenesis, and possible function. Bacteriol. Rev. 41:449-474.
Kempf, H.-J., and Wolf, G. 1989. *Erwinia herbicola* as a biocontrol agent of *Fusarium culmorum* and *Puccinia recondita* f. sp. *tritici* on wheat. Phytopathology 79:990-994.
Kusumoto, S., Yoshimura, H., Imoto, M., Shimamoto, T., and Shiba, T. 1985. Chemical synthesis of 1-dephosho derivative och *Escherichia coli* lipid A. Tetrahedron Lett. 26:909-912.
Lindow, S. E., Arny, D. C., and Upper, C. D. 1978. *Erwinia herbicola*: A bacterial ice nucleus active in increasing frost injury to corn. Phytopathology 68:523-527.
Maniatis, T. A., Fritsch, E. F., and Sambrock, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Roeder, D. L., and C. Collmer. 1985. Marker-exchange mutagenesis of a pectate lyase isozyme gene in *Erwinia chrysanthemi*. J. Bacteriol. 164:51-56.
Simon, R. 1984. High frequency mobilization of gram-negative bacterial replicons by the in vitro constructed Tn*5*-Mob transposon. Mol. Gen. Genet. 196:413-420.
Trieu-Cuot, P., and Courvalin, P. 1983. Nucleotide sequence of the *Streptococcus faecalis* gene encoding the 3′5″-aminoglucoside phosphotransferase type III. Gene 23:331-241.
Tuite, J. 1969. Page 53 in: Plant Pathological Methods: Fungi and Bacteria. Burgess, Minneapolis.
Van Outryve, M. F., Gossele, V., Gossele, F., and Swings, J. 1988. The composition of the microflora of witloof chicory seeds. Microb. Ecol. 45:1006-1011.
Winkelmann, G., Lupp, R., and Jung, G. 1980. Herbicolins—New peptide antibiotics from *Erwinia herbicola*. J. Antibiot. 33:353-358.
Wood, W. B. 1966. Host specificity of DNA produced by *Escherichia coli*: Bacterial mutations affecting the restriction and modification of DNA. J. Mol. Biol. 16:118-133.