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(54) Title: MODIFIED PHYTOPATHOGENIC BACTERIAL STRAINS AND THEIR APPLICATION FOR THE SCREENING OF MOLECULES USEFUL FOR THE PROTECTION OF CULTURES.

(57) Abstract:

THE INVENTION CONCERNS STRAINS OF PSEUDOMONAS SOLANACEARUM, PSEUDOMONAS SYRINGAE, XANTHOMONAS CAMPESTRIS OR ERWINIA AMYLOYORA, CHARACTERISED IN THAT THEY INCLUDE AT LEAST ONE CODING SEQUENCE OF A REPORTER GENE UNDER THE CONTROL OF A HRP PROMOTER GENE, SAID SEQUENCE CODING FOR A PROTEIN EXPRESSING A BIOLOGICAL OR ENZYMATIC ACTIVITY CAPABL OF BEING PARTIALLY OR TOTALLY REPRESSED IN THE PRESENCE OF MOLECULES CAPABLE OF INTERFERING WITH THE EXPRESSION OF THE HRP GENE, SAID STRAINS NOT HAVING, EITHER NATURALLY OR AFTER PRELIMINARY TREATMENT, ACTIVITY EXPRESSED BY THE CODING SEQUENCE OF THE REPORTER GENE.

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(56) Documents cited: EP 0 370 813 A2
WO 90/04037
US-J:GENE, 67 (1988), PP.287-294

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The invention relates to modified phytopathogenic bacterial strains and their application for the screening of molecules useful for the protection of cultures.

The chemical attack which aims to curb the development of a phytopathogenic microorganism or even to kill such a microorganism by acting on certain of its essential components has proved its efficacy in the protection of plants as regards numerous diseases and, notably, those caused by fungi. Nevertheless, some broad spectrum pesticides modify the balance between entire populations at the level of the ecosystem. That is why molecules having a narrow specificity and a minimal impact at the level of existing populations have been researched.

The inventors have developed a new strategy based on the research of molecules interfering with the expression of the deleterious effects of the pathogenic agent to be attacked without researching its elimination.

Recent genetic and molecular biological data have provided evidence that the hrp genes are common to large groups of phytopathogenic bacteria responsible for very diverse bacterloses in a wide variety of plant hosts.

These pathogenic genes also control the capacity for inducing a hypersensitivity reaction in non host plants

By modifying the hrp genes in Pseudomonas solanaceanum, Pseudomonas syringae (1), Xanthomonas campestris and Erwinia amylovora (2), it has been observed that it is possible to use such strains for the screening of active molecules capable of interfering with the expression of these genes (the bibliographic references are given at the end of the description).

Thus it is an object of the invention to provide new strains of P. solanacearum, P. syringae, X. campestris and E. amylovora.

It is further object to provide a process permitting the construction of said strains.

It is also an object of the invention to provide a process for the screening of substances for the purpose of detecting active materials capable susceptible of impeding the normal development of the infectious process of phytopathogenic bacteria by using said strains.

The invention relates to strains of P. solanacearum, P. syringae, X. campestris or E. amylovora characterised in that they include at least one coding sequence of a reporter gene under the control of a hrp promoter gene, said sequence coding for a protein expressing a biological or enzymatic activity, the expression of said protein may be partially or totally repressed in the presence of molecules capable of interfering with the expression of the hrp gene, said strains not having any other functional gene conferring the biological or enzymatic character coded by the reporter gene, the reporter gene being different from the gene conferring ice nucleation activity.

The invention also relates to new strains of P. solanacearum or E. amylovora, characterised in that they include at least one coding sequence of a reporter gene under the control of a hrp promoter gene, said sequence coding for a protein expressing a biological or enzymatic activity, the expression of said protein may be partially or totally repressed in the presence of molecules capable of interfering with the expression of the hrp gene, said strains not having any other functional gene conferring the biological or enzymatic character coded by the reporter gene.

These arrangements have the advantage of permitting the detection of active molecules with respect to pathogenic strains, but devoid of antibiotic activity.

By reporter gene is meant a coding sequence devoid of its own promoter and the expression of which is dependent on an exogenous promoter derived from the hrp gene. It allows one to measure the transcriptional activity of this promoter.

In one aspect, the strains according to the invention are obtained by transcriptional fusion and include, upstream of the coding sequence of the reporter gene, a ribosomal fixation and translation initiation site (Shine-Delgarno sequence).

According to another aspect, the strains according to the invention are obtained by translational fusion, the coding sequences being deprived of their ATG translation initiator codon and/or their Shine-Delgarno sequence (13).

According to an advantageous aspect of the invention, the reporter gene coding sequence codes for a protein having a biological or enzymatic activity which can be demonstrated according to classical techniques.

Among the reporter genes which may be used, one may cite the structural gene of β-galactosidase, glucuronidase, neomycin phosphotransferase, or chloramphenicol acetyltransferase, a gene conferring ice nucleation activity, or genes conferring bioluminescence.

Suitable strains for carrying out the invention are haploid strains for the hrp sequences, carrying the fusion in a hrp gene.

Other suitable strains are constituted by merodiploid strains for all or part of the hrp genes, in which the fusion of the genes is achieved in at least one of the copies of the hrp genes.

The invention also relates to a new system useful for detecting molecules for the protection of cultures, characterised in that it comprises a strain as above defined on the one hand, and a molecule capable of hindering the normal infectious process of phyto-pathogenic bacteria.

The invention also relates to the obtaining of the strains hereinbefore defined.

This process is characterised in that it comprises the steps of:

- the insertion in the hrp genes of a coding sequence of a reporter gene coding for a protein with a biological or enzymatic activity capable of being partially or totally repressed in the presence of molecules capable of interfering with the expression of a hrp gene,

- the reintroduction of the fusion thus achieved in a wild strain devoid of the activity conferred by the introduced reporter gene.

Advantageously, the reintroduction is realised either by a homologous double recombination which results in the replacement of the wild gene by the above construction, or by the introduction of a copy of the

mutated gene cloned in a vector capable of being maintained in a stable manner in the strains of P. solanacearum, P. syringae, X. campestris or E. amylovora.

The biological or enzymatic activity expressed by the strains thus modified is advantageously used in accordance with the invention to achieve in a simple manner the screening of molecules which are capable of acting on the transcription of the hrp gene.

The invention thus provides a process for the screening of substances with a view to detecting active materials capable of impeding the normal development of the infectious process in phytopathogenic bacteria, said process being characterised in that one uses a strain as hereinabove defined and in that one determines the ability of the active material to reduce the transcriptional activity of hrp genes.

One will observe that according to this process, the molecules are hindered, repressing, at least partially, the expression of the hrp gene, which thus results in a strategy having the advantage of respecting population balances.

Such a process is particularly adapted for an attack on phytopathogenic bacteria given the prohibition on the use of antibiotics in agriculture.

The determination of the activity of the materials tested involves, in particular, the detection of enzymatic activity, differential behaviour in the presence of antibiotics, measuring luminescent emission or ice nucleation activity.

By using, for example, a strain obtained by transcriptional fusion between the promotors of the hrp gene and the sequence coding for the structural gene of ß-galactosidase, in pure culture, under suitable conditions, ß-galactosidase is produced. In the presence of X-gal in the culture medium, the strain gives a blue colouration due to the hydrolysis of X-gal by ß-galactosidase. Every substance added to the culture medium and capable of specifically repressing the pathogenicity of a hrp

gene manifests itself by the disappearance or a diminution of the intensity of the colouration due to the expression of the ß-galactosidase without which bacterial growth would be affected.

The fusion between the coding sequence of the gene for ß-galactosidase and the promotor of the hrp gene may be achieved by classical in vitro or in vivo recombinant DNA techniques. The constructions useful to this end are presented in the review of Silhavy and Beckwith (14) and in the publication (5).

By way of example of substances so detected useful as active materials, one may mention casein hydrolysates or peptone. But it is clear that a large spectrum of substances may be detected by putting into practice the arrangements of the invention, by appropriate selection of a reporter gene.

The substances detected according to the invention enable one to effectively contend not only with the bacterial blighting due to P. solanacearum, but also against other diseases caused by the pathovars P. syringae and X. campestris together or by E. amylovora.

Other characteristics and advantages of the invention will be apparent from the following description with reference to the Examples and to the Figures 1 and 2,

- Figure 1 represents in the region pVir2 and pAFE8, groups with transcriptional activity such that they may be defined by means of constructions of the invention, and

- Figure 2, the kinetics of the induction of the activity of ß-galactosidase measured in a mutant of the invention in the presence of vegetable extracts.

EXAMPLE 1: P. SOLANACEARUM STRAIN MODIFIED BY THE INSERTION OF TRANSPOSON Tn5-B20.

*MATERIALS AND METHODS

1/ Bacterial strains and plasmids used.

The following table indicates the essential characteristics of the strains and plasmids used.

TABLE

BACTERIAL STRAINS AND PLASMIDS

Designation of the strain	Genotype and/or essential characters; construction	origin/ reference
E. coli		
C600 Sm^r	lambda$^-$,F$^-$,*thi*,*thr*,*leu* *rpsL*, *sup*E44,*hsd*R,*hsd*M$^+$	(11)
S17-1	*pro*, *hsd*R,*rec*A,RP4-2	(15)
K12(pRK2013)	lambda$^-$,P$^-$,km^r of pRK2013	(12)
P. solanacearum		
GMI 1000	wild strain, pathogen of tomato pathotype 1, Biovar 4	(3)

Plasmids	characterisation	
pRK2013	TraRK2$^+$, delta (*rep* RK2), *rep*E1$^+$; Km^r	(2)
pVir2	derivative of pLAFR3 carrying a part of the hrp genes of P. solanacearum	(4)
pAFE8	plasmid carrying an insert of 25kb overlapping the 3.5kb region at the left end of the insert of pVir2	

2/ Cultures and preservation of bacterial strains.

The strains of P. solanacearum are cultivated at 30°C:

The media used and their compositions given in grams per litre are medium B (peptone 10 yeast extract 1, casein hydrolysate 1), BG (identical to B and supplemented with glucose : 5), BGT (identical to BG and supplemented with triphenyltetrazolium chloride : 0.05), MMG ($FeSO_4$, $7H_2O$: 1,25.10^{-4}; $(NH_4)_2SO_4$: 0.5 $MgSO_4$, $7H_2O$: 0.05 KOH (1ON): 1.75 /ml; KH_2PO_4: 3.4; glucose 2).

The strains of E. coli are cultivated at 37°C in medium L containing in grams per litre (yeast extract :5; Bactotryptone: 10; NaCl : 10).

The media are supplemented as required with the following antibiotics: streptomycin (Sm) 200 µg/ml; nalidixic acid (Nal) 25 µg/ml; tetracycline (Tc) 10µg/ml and kanamycin (Km) 25 µg/ml. The media are optionally solidified by the addition of agar at 15 g/l.

3/ Bacterial conjugation

The donor and recipient strains as well as the "helper" K12(pRK2013) are cultivated for approximately 14 hours in non-selective liquid nutritive medium. The precultures of the donor and helper strains are then diluted in fresh liquid nutritive medium. When the O.D. (650nm) of these new cultures reaches 0.2 (10^8 bacteria/ml), 0.5ml of the latter and 0.5 ml of the culture of the recipient strain are mixed and filtered on a Millipore filter with 0.45 µm diameter pores.

The filters are then placed on dishes of solid nutritive medium, the bacteria being above the membranes. The nature of the nutritive medium and the incubation conditions depend on the bacteria involved in the crossing:-

-for crossings between strains of E. coli, the filters are placed on medium L and incubated at 37°C for 4 hours,

-when one of the crossing partners is P. solanacearum, the filters are placed on medium BG and incubated for from 4 to 18 hours at 30°C.

Two controls are set up by separately incubating the crossing partners under the same conditions.

The bacteria are then resuspended in 3ml of nutritive medium and appropriate dilutions of the suspension are spread out on selective medium.

4/ Mutagenesis by the transposon Tn5-B20

The transposon Tn5-B20 (5) was used for the mutagenesis of the recombinant cosmids pVir2 and pAFE8.

The aforementioned transposon is inserted in a lambda phage which carries an amber mutation. A lysate of this phage was produced by the infection of the strain C600 (su^+=supE), according to the protocol of lysate preparation on a petri dish described in (6). The mutagenesis is achieved by infecting the E. coli strain S17-1 including the plasmid pVir2 or pAFE8 with this phage lysate.

To this end, the strains are cultivate at 37°C in medium L containing 0.2% of maltose. When the O.D. (650nm) of the culture reaches 0.8 (10^9 bacteria/ml) it is infected by the phage lysate at a multiplicity of infection of 0.5 phage per bacterium at 37°C for a period of 2 hours.

The cells are then subjected to two successive washings so as to rid them of phage and are then spread out on the medium L SmTcKm, which allows one to select the strains carrying a copy of the transposon. The Km^r mutants are crossed en masse with the recipient strain C2110 Nal^r in the presence of the strain K-12 (pRK2013), the product of the crossing is then spread on the medium L NalTcKm and incubated at 37°C which allows one to select the transfer of cosmids carrying the transposon.

5/ Determination of ß-galactosidase activity.

ß-galactosidase activity was carried out according to the protocol described by Miller (7) but modified by carrying out the enzymatic test in a 1.5ml Eppendorf tube.

The ß-galactosidase activity is determined in bacterial cultures whose optical density (O.D. 600nm) must be between 0.2 and 0.8. After having measured the O.D. of the culture at 600 nm, 375 µl of the culture are mixed with an equal volume of buffer Z (0.06M Na_2HPO_4; 0.04M NaH_2PO_4; 0.01M KCl; 0.05M $MgSO_4$; 0.05M ß-mercaptoethanol; pH: 7.0) in a 1.5ml Eppendorf tube. The cells are lysed by adding 100 µl of chloroform and 50 µl of 0.1% SDS. After 5 minutes incubation at 28°C 150 µl ONPG (4mg/ml) are added to start the reaction. When the yellowing of the reaction medium intensifies, the reaction is stopped by the addition of 375 µl of 1M Na_2CO_3. The optical density of the reaction medium,cleared of cellular debris by centrifugation (120,000g, 3 min), is measured at 420 nm and 550 nm. The O.D. at 420 nm allows one to measure the quantity of o-nitrophenol formed. The O.D. at 550 nm allows one to correct the error on the reading at 420 nm generated by the presence of cellular debris.

The units of ß-galactosidase activity are calculated by applying the formula presented below:

Units= 1,000 O.D. (420 -1.75 . O.D. 550)/ t .v . O.D.600

The O.D. 600 reflects the bacterial concentration before the test

t: reaction time expressed in minutes;

v: volume of culture used for the test, expressed in ml.

6/ Tests for pathogenicity in tomatoes.

The surface of tomato seeds (Marmande cultivar, supplied by Société Vilmorin, Beaufort en Vallée, France) is sterilised at 30°C for 45 minutes in a 7% solution of calcium hypochlorite (w/v). The seeds are then rinsed abundantly with deionised sterile water before being placed on dishes of medium B. The dishes are incubated for 60 hours at 30°C, which enables one to obtain germination of the seeds and controlled sterility. The germinated seeds are transferred in pairs into sterile test tubes (22 mm in diameter) which contain 1.5g of vermiculite and 6ml of nutritive solution (5mM KNO_3; 2mM KH_2PO_4; 2mM $Ca(NO_3)_2$; 1.5mM $MgSO_4$; 24µM H_3BO_4; 8.9µM $MnSO_4$; 3.1µM $ZnSO_4$; 1µM $CuSO_4$; 0.1µM K_2MoO_4 and 0.1mM $FeSO_4$ complexed by 0.1mM EDTA). The tubes are placed in a culture chamber (16h, 3600 lux, 30°C and 85% relative humidity; 8h in the dark, 28°C and 85% relative humidity). After 4 days the completely unfolded cotyledenous plants are innoculated under asceptic conditions by adding to each tube 1ml of water containing 10^7-10^8 bacteria to be tested. Each bacterial strain is thus tested on 4 tubes. Also, for each experiment, 4 tubes are innoculated with the strian GMI1000 and four others with sterile water by way of control.

The mutants derived from the strain GMI1000 which killed all of the plantlets 15 days after innoculation are considered as being pathogens. The mutants which did not induce any symptoms 21 days after innoculation are considered as non pathogens. Finally, the mutants which killed only part of the innoculated tomato plantlets or which bring about a delay in the appearance of the symptoms of the disease or, indeed, a significant slowing down of growth of the plantlets are considered as hypoaggressive.

7/ Tests for HR(hypersensitive reaction) in tobacco

Tobacco plants (Bottom Special cultivar) are cultivated in a greenhouse (18+2°C, 16 h of light, 8 h in the dark) in 15 cm pots containing a mixture(1:1, v/v) of peat and horticultural compost.

Nine week old tobacco plants are innoculated with mutants derived from the strain GMI1000 according to the protocol described in (8).

The cell cultures in the exponential phase of growth (10^8 bacteria /ml) of strains to be tested are harvested by centrifugation (3000 g, 10 min.) and resuspended in sterile water so as to have 10^7 bacteria / ml. The suspensions are infiltrated into the tissue between two veins of completely unfolded young leaves. One leaf may thus receive 3 or 4 independent infiltrations. The innoculated tobacco plants are placed in a culture chamber (16 h, 3600 lux, 30°C and 85% relative humidity; 8 h in the dark, 28°C and 85% relative humidity). The results are noted 48 and 60 hours after the infiltration.

8/ Production of exudates of tomato roots

The method developed enables one to obtain 300 ml of rood exudate solution from 600 tomato seeds (Marmande cultivar).

After sterilisation and rinsing (c.f. paragraph 6), the tomato seeds are placed in a Petri dish (13.5 cm in diameter) and then covered with a 0.7 % supercooled aqueous solution of soft agar. The Petri dish is lightly agitated to ensure a uniform distribution of the seeds in the agar. After solidification, the agar is cut up and the pieces are transferred asceptically into a beaker on a grill which rests at the surface of 300 ml of sterile deionised water. The beaker is then placed in a culture chamber (16h, 3600 lux, 30°C and 85% relative humidity; 8h in the dark, 28°C and 85% relative humidity).

These conditions enable one to obtain, from the second day of incubation, plantlets the roots of which are developing insterile water.

After 5 days of incubation, when the cotyledons of the plantlets are completely unfolded, the 300 ml of water are collected and filtered on a Millipore membrane with 0.45µm diameter pores. 200µl of the root exudate are spread out on a dish of medium B and then incubated for several days at 30°C to test the sterility thereof. The remainder of the solution is stored at -20°C.

9/ Tobacco extracts

The tobacco extracts had been prepared in the laboratory from cell suspensions of medullary parenchyma of Nicotiana tabacum cultivar Wisconsin 38 (progeny 19-3).

The tobacco cells are cultivated according to the method described in (9) and are subcultured everyday for 14 days in fresh JPL medium. To prepare the tobacco extract, the cellular suspension obtained 14 days after the subculture (stationary phase of growth) were successively filtered on a sintered glass filter No.1 and on a Millipore filter having 0.45 µm diameter pores. The sterility of the filtrate was tested by plating a part thereof on medium B and by incubating the Petri dishes for 4 days at 30°C. The tobacco extract thus produced is stored at -20°C.

10/ Induction of the expression of hrp genes

Derivatives of the GMI1000 strain carrying an insertion of transposon Tn5-B20 in the hrp region were cultivated in minimal MMG medium. When the cultures entered the exponential phase of growth they were diluted 100 or 50 times in 5ml of fresh MMG medium and in 4 ml of MMG medium containing 1ml of tobacco extract or tomato root exudate. These new cultures carried out in 22mm test tubes were then incubated with shaking at 30°C for a period of 20 hours before the determination of ß-galactosidase activity.

11/ Molecular biology techniques

The techniques used (extraction of plasmids, analysis of restriction fragments, preparation of radioactive probes, hybridisation on nylon membrane and the like) are those currently employed and already described in (10).

*RESULTS

1. CHARACTERISATION OF THE hrp GROUP OF GENES.

Each clone carrying an insertion of Tn5-B20 at the level of the insert of cosmids pVir2 or pAFE8 was conjugated with the GMI1000 strain devoid of ß-galactosidase activity so as to introduce each of the insertions in the genome of this strain by the marker exchange technique. After conjugation, the transconjugants of P. solanacearum resistant to kanamycin were selected. All of the clones thus isolated were found to be sensitive to tetracycline indicating that the cosmid has been lost in these transconjugants and that the transposon is itself certainly integrated in the genome of these strains by a double occurrence of homologous recombination.

The former has been verified by a hybridisation of the genomic DNA of these transconjugants with probes constituted by the plasmids pVir2 and pAFE8.

The recombinant strains derived from GMI1000 were individually innoculated on axenic tomatoes and infiltrated into tobacco leaves.

The localisation of the insertion site of Tn5-B20 in each clone obtained was determined by analysis of the size of the restriction fragments. These sites are shown in Figure 1.

This Figure represents the localisation of the insertions Tn5-B20 in the genomic regions carried by the cosmids pVir2 and pAFE8 and the level

of ß-galactosidase activity and the properties in plants of corresponding mutants.

The insertions of which the sequences of the lacZ gene are orientated from left to right are represented above the genetic map of the strain GMI1000. The insertions whose lacZ gene sequences are orientated from right to left are represented below.

The properties in plants are indicated by the following symbols:

□ , ■ identical reaction to the strain GMI1000;

○ , ● partial or total loss of the capacity to induce HR in tobacco and pathogenicity in the tomato. The frame hatched by the fine line delimits the group of hrp genes.

The insertions conferring a ß-galactosidase activity greater than 5 units are indicated by the closed symbols: ■ , ●)

The insertions inducing a ß-galactosidase activity less than 5 units are indicated by the open symbols : □ , ○)

The horizontal arrows situated under the genetic map represent the hrp transcription groups.

The direction of the arrow indicates the direction of transcription of each of the groups. The number of each transcription group is represented above the arrow. The level of ß-galactosidase activity measured for the insertion allowing one to find these groups is reported under each arrow (200;> 200 units ; 150: from 120 to 170 units; 100: from 70 to 120 units; 50: from 30 to 70 units; 20: 18 to 25 units; 15: 12 to 16 units).

2.EXPRESSION OF hrp GENES.

The transposon Tn5-B20 creates transcriptional fusions between the lacZ gene deprived of its own promotor and the genes into which it is inserted. This property has been used to characterise the trascribed hrp sequences, their direction of transcription, and the conditions under which they express themselves. The expression at the level of an insertion site is tested by measuring the ß-galactosidase activity, the appearance of a significant ß-galactosidase activity in a derivative of the strain GMI1000 carrying an insertion of Tn5-B20 in the genome indicates that the sequences of the lacZ gene are situated downstream of a promotor in the transcribed region and in the same transcriptional orientation.

The ß-galactosidase activity produced by each of the derivatives of the GMI1000 strain carrying an insertion of Tn5-B20 and cultivated in minimal medium has been measured. It has been observed that the level of ß-galactosidase activity produced by these derivatives was variable and that it was possible to classify them into 6 groups according to the level of ß-galactosidase activity obtained:

-for the first group of derivatives, the level of ß-galactosidase activity measured is greater than or equal to 200 units;

-for the second, this level is of the order of 150 units(from 120 to 170 units);

-for the third, this level is of the order of 100 units (from 70 to 120 units);

-for the fourth, this level is in the region of 50 units (30 to 70 units);

-for the fifth, this level is between 15 and 20 units;

-finally, for the sixth group, which corresponds to a little less than half of the strains tested, this level is less than 5 units.

The last group corresponds, probably, to insertions whose lacZ gene is not transcribed either because they are:

-situated outside the transcribed region,

-localised in a transcribed region but orientated (relative to the lacZ gene) in the opposite direction to the direction of transcription;

-inserted in the correct orientation at the level of the transcribed region but which is not expressed under the experimental conditions used.

In contrast, mutants belonging to other activity level groups certainly carry insertions localised at the level of regions transcribed in MMG.

By linking for each mutant the level of β-galactsidase activity conferred by the insertion with its position and its orientation on the physical map of the region studied, a correlation has been observed between these three parameters.

These results confirm that the Tn5-B20 transposon is utilisable for detecting the transcription of genes in P. solanacearum. The existence of several transcribed regions at the level of the group of hrp genes has been established. The orientation of transposons within these different regions has allowed one to determine their direction of transcription. Based on the levels of activity induced by these insertions introduced in these different regions, on their direction of transcription and on the phenotype in plants that they confer on the strains, 9 different transcription groups have been distinguished (Figure 1). The groups hrpI and II which are contiguous and transcribed in the same orientation (from right to left =rl) have been separated into two different groups because the insertions of group hrpI induce a β-galactosidase activity of 50 units while the insertions of the group hrp II induce an activity of 200 units. This separation is confirmed by the fact that the insertions 1484, 1457 and 1443 situated at the level of the 3' end of the transcript (potential) of group hrpII and at the 5' end of the transcript (potential)

of group hrpI induce a wild phenotype whereas the adjacent insertions of groups hrpI and II induce a hrp⁻ phenotype.

Consequently these three insertions suggest that the groups hrp I and II are separated by a non-coding region and do not belong to the same units of transcription. The insertions 1484 and 1457 do not affect the pathogenicity even though they are situated at the 3' end of the hrpII transcription group. Nevertheless, they confer on the strain a capacity to produce ß-galactosidase at a level equivalent to that of other fusions achieved in the hrpII region. This may be explained by the fact that the Tn5-B20 produces transcriptional fusions and that these insertions are localised upstream of the termination sequences of the hrpII region and downstream of the coding sequences.

Moreover, in the case of the hrp VII and VIII transcription groups which are contiguous and have an identical direction of transcription bu different expression levels, the insertion 1421 induces a wild phenotype confirming the existence of two distinct trancription groups. The fact that this insertion, situated at the level of the 3' end of the transcript (potential) of the hrpVII group and whose orientation (relative to the lacZ gene) is analogous to the direction of transcription of this transcription group produces a ß-galactosidase activity similar to that produced by the insertions localised in this group may be justified for the same reasons as those previously invoked for the insertions 1484 and 1457 of the hrpII transcription group.

The insertions 1503 and 1415 which do not alter the pathogenicity, demonstrate the existence of a non-transcribed region between the hrpVI and VII transcription groups whose directions of transcription are opposed. In this case also results obtained by analysis of the galactosidase activities and the phenotypes in plants agree.

3. STUDIES ON THE REGULATION OF THE hrp GENE: INDUCTION BY PLANT EXTRACTS.

To study the regulation of the transcription of hrp genes, derivatives of the GMI1000 strain carrying a Tn5-B20 insertion were cultivated in MMG in the presence and in the absence of tomato root exudates or of tobacco cell culture filtrates. After 20 hours of contact of the bacteria with the extracts, the relationship between ß-galactosidase activity produced in the MMG containing the tomato or tobacco extracts and the activity produced in the MMG in the absence of such extracts was determined for each insertion. This experiment was repeated 3 times for each insertion.

For all of the insertions localised in the transcription groups hrpI, II IV, V, VI, VII, VIII and IX such that the orientation of the lacZ gene corresponds to the direction of transcription of each of these groups, it was observed that the relationship resulting from the experiments was between 2 and 5 independent of the vegetable extract used (tomato or tobacco).

In contrast, for the insertions situated at the level of these groups but of opposite orientation, no increase in ß-galactosidase activity was observed in the presence of plant extracts. For the insertions localised at the level of the hrpIII transcription group, the ratio is always in the region of 1 independent of the orientation of the insertions.

It has been considered that the insertions for which the ratio is greater than 2 indicates an activation of transcription by the plant extracts. These results show that the expression of the genes situated in the transcription groups hrpI,II, IV, V, VI, VII, VIII and IX is induced by extracts of tomato and tobacco. The level of induction revealed in the course of these experiments was shown to be the same for all transcription groups and no difference was observed in the level of induction as a function of the type of vegetable extract used.

In contrast, the level of transcription for the hrpIII group was not affected by these extracts. This transcription group is the group which shows the lowest ß-galactosidase activity (15 units) in MMG. This low level was measured for all the insertions present in this region and appeared significantly different from the level detected for the insertions for which the lacZ gene is not transcribed (<5 units). It is thus considered as a transcription group.

The overall results obtained in the course of these studies indicate that the expression of certain hrp genes is positively regulated by one or more plant products.

Figure 2 (units of ß-galactosidase as a function of time in hours after subculture in the presence or absence of tomato exudates) shows the kinetic induction of ß-galactosidase activity for the mutant hrp⁻, which carries the insertion 1388, cultivated in MMG (curve 0 - 0) or in the absence of tomato root exudate. The curves presented show that the increase in the ß-galactosidase activity induced by this extract is detectable after 9 hours and that it reaches a plateau after 12 hours.

4. NEGATIVE REGULATION OF hrp GENES

On cultivating the mutant hrp⁻ carrying the insertion 1388 in liquid medium B, it was observed that the ß-galactosidase activity produced by the bacterial cells was less that 10 units whereas in MMG the insertion induced an activity of approximately 150 units. This result suggests that the expression of the hrpVII transcription group is either repressed in medium B or specifically induced in MMG. Similar results have been obtained with the insertions localised in the transcription groups as a whole.

To determine if the transcription of these hrp genes is induced in MMG or repressed in medium B, the mutant carrying the insertion 1388 was cultivated on the one hand in complete medium B supplemented by each of the constituents included in the composition of MMG and on the other hand in MMG supplemented by each of the constituents of MMG.

In the two cases, the concentration of the added constituents was equal to that which was found in the original medium. The ß-glactosidase activity produced by the mutant in each of these cultures was then measured. The level of activity in medium B was not modified by the addition of the constituents of MMG. In contrast, the addition of casein hydrolysate or peptone in MMG brought about a lowering of the ß-galactosidase activity produced by the mutant. The level detected was then comparable to that obtained in medium B. These results show that the expression of hrp genes (belonging to group hrpVII) is repressed in medium B and that the casein hydrolysates or the peptone are responsible for this repression.

Another series of experiments has shown that the addition of tomato or tobacco extract to the medium B does not overcome the repression of the expression due to casein hydrolysates or peptone.

REFERENCES BIBLIOGRAPHIQUES

1. LINDGREN, P.B., PEET R.C., et N.J. PANOPOULOS, J. Bacteriol. 168:512-522, 1986

2. STEINBERGER E.M. et S.V. BEER. MPMI 1:135-144, 1988

3. MESSAGE, B., BOISTARD, P., PITRAT, M., SCHMIT, J. et C. BOUCHER, Proc. 4th Int. Conf. Plant Pathog. Bact. Station de Pathologie Végétale INRA (ed.) INRA, Beaucouzé, Angers, France, p.823-833, 1978

4. BOUCHER, C.A., VAN GIJSEGEM, F., BARBERIS, P.A., ARLAT M. et ZISCHEK, C. J. Bacteriol. 169:5626-5632, 1987

5. KELLER M., MULLER, P., SIMON R., et A. PUHLER, MPMI 1:267-274, 1988

6. SILHAVY, T. J., BERMAN, M.L. et ENQUIST, L.W., Cold Spring Harbor Laboratory, Cold Spring Harbor, 1984

7. MILLER, J. Cold Spring Harbor Laboratory, Cold Spring Harbor N.Y., 1972

8. KLEMENT, Z. Nature 199:299-300, 1963

9. JOUANNEAU, J.P. et PEAUD-LENOEL, Physiol. Plant 20:834-850, 1967

10. MANIATIS, T., FRITSCH, E.F. et J. SAMBROOK, Cold Spring Harbor Laboratory Press, New York, 1982

11. JULLIOT, J.S. et P. BOISTARD, Molec. Gen. Genet. 173:289-298, 1979

12. FIGURSKI, D.H. et D.R. HELINSKI, Proc. Natl. Acad. Sci. USA 76:1648-1652, 1979

13. SHINE J. et L. DELGARNO, Proc. Natl. Acad. Sci. U.S.A. 71:1342-1346, 1974

14. SILHAVY T.J. et J.R. BECKWITH, Microbiol. Rev. 49:398-418, 1985

15. SIMON R., PRIEFER U., et A. PUHLER, Molecular Genetics of the Bacteria Plant Interactive, A. Pühler éditeur. Springer Verlag p.98-106, 1983

CLAIMS

1. Strains of Pseudomonas solanacearum, Pseudomonas syringae, Xanthomonas campestris or Erwinia amylovora, characterised in that they include the coding sequence of a reporter gene under the control of a hrp promoter gene, said sequence coding for a protein expressing a biological or enzymatic activity, the expression of said protein may be partially or totally repressed in the presence of molecules capable of interfering with the expression of the hrp gene, said strains not having any other functional gene conferring the biological or enzymatic character coded by the reporter gene, the reporter gene being different from the gene conferring ice nucleation activity.

2. Strains of Pseudomonas solanacearum or Erwinia amylovora, characterised in that they include the coding sequence of a reporter gene under the control of a hrp promoter gene, said sequence coding for a protein expressing a biological or enzymatic activity, the expression of said protein may be partially or totally repressed in the presence of molecules capable of interfering with the expression of the hrp gene, said strains not having any other functional gene conferring the biological or enzymatic character coded by the reporter gene.

3. Strains according to either of claims 1 or 2, characterised in that they are obtained by transcriptional fusion and include upstream of the coding sequence of the reporter gene a ribosomal fixation and translation initiation site (Shine-Delgarno sequence).

4. Strains according to either of claims 1 or 2, characterised in that they are obtained by translational fusion and in that the coding sequence is deprived of its ATG translation initiator codon and/or its Shine-Delgarno sequence.

5. Strains according to any one of claims 1, 3 or 4, characterised in that the coding sequence of the reporter gene is selected from the structural gene of β-galactosidase, glucuronidase, neomycin phosphotransferase, chloramphenicol acetyltransferase, or genes conferring bioluminescence.

6. Strains according to any one of claims 2-5, characterised in that the coding sequence of the reporter gene is selected from the structural gene of β-galactosidase, glucuronidase, neomycin phosphotransferase, or chloramphenicol acetyltransferase, a gene conferring ice nucleation activity, or genes conferring bioluminescence.

7. Strains according to any one of claims 1-6, characterised in that they are haploid strains for the hrp sequences, carrying the fusion in the hrp gene.

8. Strains according to any one of claims 1-6, characterised in that they are merodiploid strains for some or all of the hrp genes, in which the fusion of genes is acheived in at least one of the copies of the hrp genes.

9. System useful for detecting molecules for protecting cultures, characterised in that it comprises a strain according to either of claims 1 or 2 on the one hand, and a molecule capable of hindering the infectious process of phytopathogenic bacteria.

10. Process of obtaining a strain according to any one of claims 1-8, characterised by the steps of:

- the insertion in the hrp genes of a coding sequence of a reporter gene coding for a protein with a biological or enzymatic activity capable of being partially or totally repressed in the presence of molecules capable of interfering with the expression of a hrp gene,

- the reintroduction of the fusion thus achieved in a wild strain devoid of the activity conferred by the introduced reporter gene.

11. Process according to claim 10, characterised in that the reintroduction is realised either by a homologous double recombination which results in the replacement of the wild gene by the above construction, or by the introduction of a copy of the mutated gene cloned in a vector capable of being maintained in a stable manner in the strains of P. solanacearum, P. syringae, X. campestris or E. amylovora.

12. Process for the screening of substances for the detection of active materials capable of impeding the normal development of the infectious process in phytopathogenic bacteria, characterised in that there is used a strain according to any one of claims 1-8, and that one determines the ability of the active material to reduce the transcriptional activity of hrp genes.

DATED THIS 8 DAY OF JUNE 1990

FISHER CORMACK & BOTHA
Patent Agents for the Applicants

ABSTRACT

Modified phytopathogenic bacterial strains and their application for the screening of molecules useful for the protection of cultures.

The invention concerns strains of Pseudomonas solanacearum, Pseudomonas syringae, Xanthomonas campestris or Erwinia amylovora, characterised in that they include at least one coding sequence of a reporter gene under the control of a hrp promotor gene, said sequence coding for a protein expressing a biological or enzymatic activity capable of being partially or totally repressed in the presence of molecules capable of interfering with the expression of the hrp gene, said strains not having, either naturally or after preliminary treatment, activity expressed by the coding sequence of the reporter gene.

(No Figure).

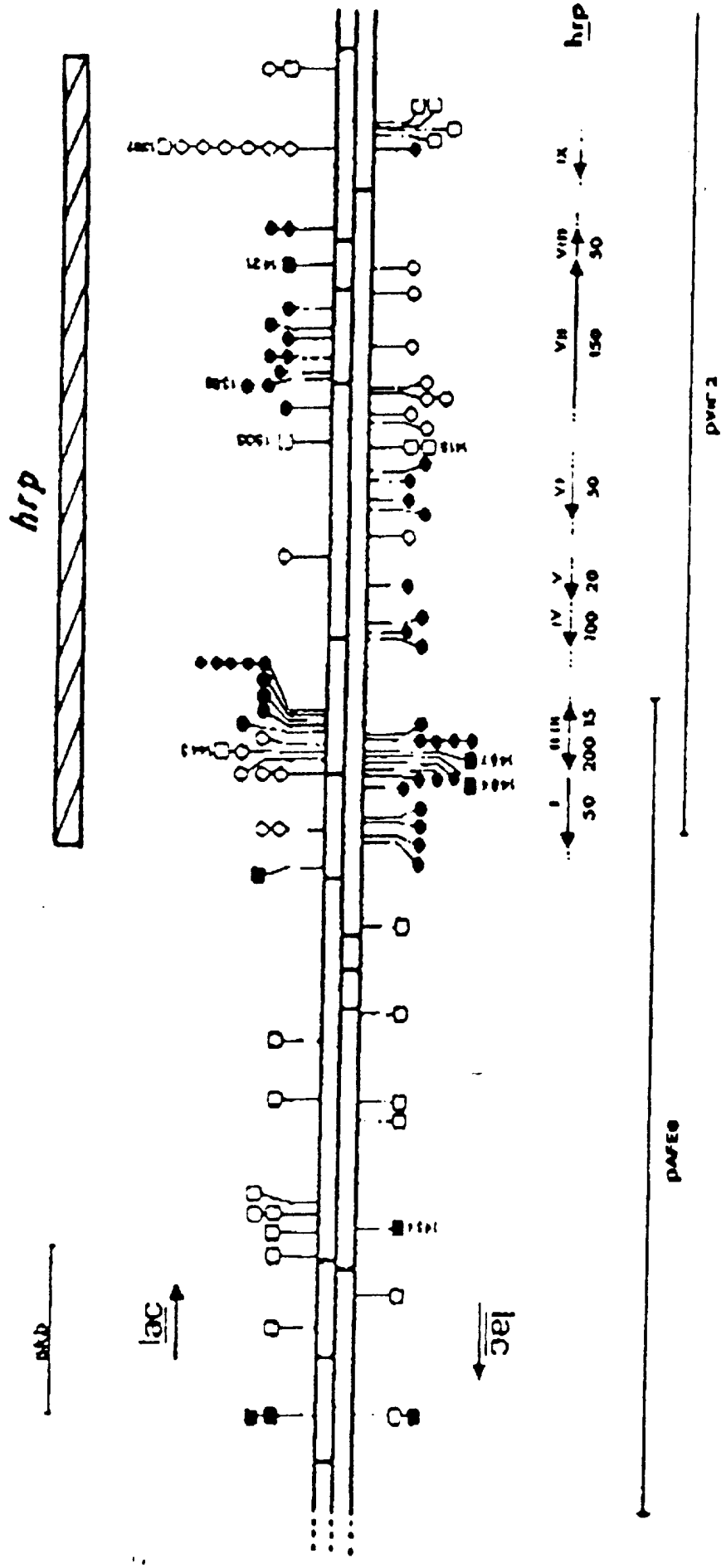

FIGURE 1

FIGURE 2