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MOLECULAR CLONING AND EXPRESSION IN STREPTOMYCES LIVIDANS OF A HYGROMYCIN B PHOSPHOTRANSFERASE GENE FROM STREPTOMYCES HYGROSCOPICUS

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The gene encoding the phosphotransferase enzyme that modifies hygromycin B in its producing organism Streptomyces hygroscopicus, has been cloned in the Streptomyces vector pIJ41. Two plasmids, pFM4 and pFM6, containing 2.1 and 19.6 kb inserts of Streptomyces hygroscopicus DNA, respectively, which express the modifying enzyme, have been isolated. A 3.1 kb PstI restriction fragment from pFM4 was inserted in the Streptomyces vector pIJ350 and the resulting plasmids, pMZ11.1 and pMZ11.2, express the hygromycin B-resistance phenotype. The utility of this dominant marker for cloning experiments is discussed in the text.

Hygromycin B is an aminocyclitol antibiotic that specifically inhibits protein synthesis at the translocation step on 70S and 80S ribosomes (1,2). The drug also induces misreading both "in vivo" and "in vitro" (1-3). Hygromycin B is a potent inhibitor of growth in gram (+) and gram (-) bacteria and certain eukaryots yeast, plant and animal cells. Because of its antihelminthic properties in farm animals the antibiotic has found use in veterinary medicine (4).

Hygromycin B, which is produced by Streptomyces hygroscopicus, is phosphorylated by cell-free extracts of this organism (4,5). This finding indicates that S. hygroscopicus DNA contains a gene encoding a phosphorylating enzyme that inactivates the antibiotic. The autoimmunity mechanisms of antibiotic-producing Streptomyces often involves the enzymatic inactivation of the relevant drugs (7-9). The genetic determinants for several of these modifying enzymes have been cloned and employed as dominant selective markers for Streptomyces cloning vectors (7). Recently, two research groups described independently the finding of R factor-determined resistance to hygromycin B in Escherichia coli. Again the

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inactivation involved the phosphorylation of hygromycin B (10,11). The genes coding for this activity have been cloned and the nucleotide sequence of one of them has been determined (10).

In the present communication we report the cloning and expression in Streptomyces lividans of the gene that determines a hygromycin B-inactivating phosphotransferase in S. hygroscopicus and its inclusion in a Streptomyces plasmid as a selectable marker for cloning experiments.

MATERIALS AND METHODS

Streptomyces hygroscopicus NRRL 2387 produces hygromycin B and was provided by D.T. Wicklow. S. lividans 1326, S. lividans TC52, carrying plasmid pIJ41 (Fig 1) and S. lividans TK128, carrying plasmid pIJ350 (Fig 1) were provided by D.A. Hopwood.

Growth media was liquid YEME supplemented with 34% sucrose (12). Solid media was R2YE agar (12). Chromosomal and plasmid DNAs were prepared from liquid culture exactly as described (12). Standard procedures were used for cloning S. hygroscopicus DNA (13-15). The presence of covalently-closed-circular DNA (cccDNA) in the Streptomyces transformants was detected by electrophoresis in 1% agarose of DNA samples obtained by the mini-prep method (16).

Hygromycin B phosphotransferase (HPH) and neomycin phosphotransferase were assayed by 5'[γ]-^{32}P - ATP-dependent phosphorylation of the relevant antibiotic using the phosphocellulose paper technique (17). Cells from cultures grown up to late log phase were broken by grinding with two volumes of alumina and extracted with one volume of 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NH_4Cl, 0.5 mM dithiothreitol buffer. After centrifugation at 150000 x g for 90 min the supernatant was used as a source of enzyme. Reactions were allowed to take place for 20 min at 30°C. Biological activity of hygromycin B and phosphohygromycin B were tested by the paper disc method using Bacillus subtilis as the sensitive organism.

Restriction enzymes and T4 DNA ligase were purchased from Ingenasa (Spain), New England Biolabs and Bethesda Research Laboratories and used indistinctly. [γ]-^{32}P - ATP was obtained from Amersham. All other chemicals were obtained from Merck and Sigma.

RESULTS AND DISCUSSION

We initially tested the response to both hygromycin B and neomycin of the different Streptomyces species used in this work. S. lividans 1326, the host for transformation, and S. lividans TC52, which carries plasmid pIJ41 (Fig 1), were sensitive to 50 µg/ml hygromycin B on R2YE plates. S. hygroscopicus NRRL 2387, produces hygromycin B and is resistant to this antibiotic at concentrations higher than 1000 µg/ml on R2YE plates. As previously found by Leboul and Davies (5) homogenates from S. hygroscopicus NRRL 2387 contained a high HPH activity

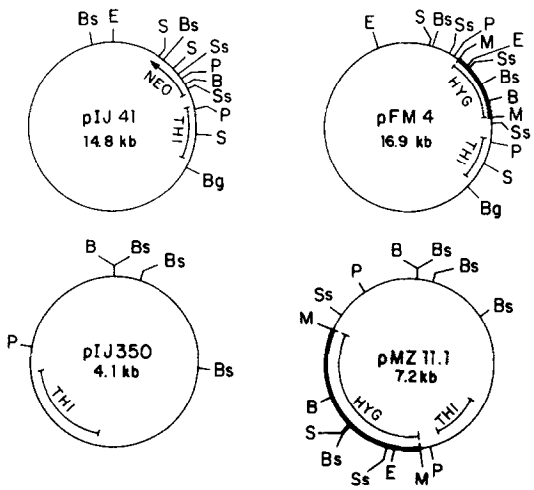

Fig 1.- Restriction endonuclease maps of pIJ41 and pIJ350 (12,14) and their derivatives pFM4 and pMZ10.1, respectively. Restriction sites were determined by comparison of single and double digestion patterns. The fragment indicated HYG corresponds to the 2.1 kb DNA insertion from S. hygroscopicus. The meaning of the simbols is: B,BamHI; Bg,BglII; Bs,BstEII; E,EcoRI; M,MboI; P,PstI; S,SalI and Ss,SstI.

(Table 1) which did not affect neomycin (not shown). On the other hand, the pIJ41 encoded neomycin phosphotransferase activity did not modify hygromycin B (not shown). Both S. lividans 1326 and S. hygroscopicus NRRL 2387 were sensitive to 5 μg/ml thiostrepton and 20 μg/ml neomycin. From these results it is clear that resistance or sensitivity to hygromycin B and neomycin can be expressed and assayed independently.

TABLE I.- Phenotypic characteristics of the different Streptomyces.

Streptomyces	Plasmid (kb)	HPH activity nmol ^{32}P / min x mg protein	Resistance to hygromycin B μg/ml
S. lividans 1322		0.0	<50
S. lividans TC52	pIJ41 (18.8)	0.0	<50
S. hygroscopicus NRRL 2387	-	1.1	>1000
S. lividans Tr1	-	0.9	>1000
S. lividans Tr4	pFM4 (16.9)	1.0	>1000
S. lividans Tr6	pFM6 (34.4)	0.7	>1000

Size of plasmid pIJ41 was taken from ref. 1. Sizes of plasmids pFM4 and pFM6 were deduced from the sizes of endonucleases cleavage fragments as determined by agarose gel electrophoresis. HPH activity was assayed as indicated in Material and Methods. Resistence to hygromycin B was determined on R2YE agar plates containing a range of hygromycin B concentrations from 0 to 1000 ug/ml. (-) indicates that the presence of plasmid DNA could not be detected.

In order to clone the gene coding for the HPH enzyme, DNA from S. hygroscopicus was partially digested with the restriction endonuclease MboI to give 2-10 kb fragments and then ligated to BamHI cleaved pIJ41 with T4 DNA ligase. The ligation mixture was used to transform S. lividans and three S. lividans transformants, (Tr1, Tr4 and Tr6) were selected as resistant to both hygromycin B and thiostrepton and sensitive to neomycin. They were then examined for the presence of both cccDNA and HPH activity. Extracts from the three transformants showed levels of enzymatic activity similar to those in extracts from the producing organism (Table 1). Therefore it can be concluded that S. lividans Tr1, Tr4 and Tr6 contain and express the hygromycin B phosphotransferase gene (HYG) from S. hygroscopicus. The inactivation of hygromycin B by phosphorylation was shown by the lack of activity of hygromycin B after incubation with the three homogenates, assayed by the disc method versus B. subtilis. This result suggests that S. hygroscopicus protects itself versus the toxic effects of hygromycin B, at least in part, by phosphorylating the antibiotic.

Analysis by agarose gel electrophoresis of intact DNA from mini-preps indicated the presence of plasmid DNA in transformants S. lividans Tr4 and Tr6 (Fig. 2). However transformant S. lividans Tr1 lacked any DNA band migrating differently from chromosomal DNA (not shown). Analysis of DNA from these three transformants in CsCl gradients showed plasmid DNA bands in the case of S. lividans Tr4 and Tr6 but no evidence for plasmid DNA was obtained with preparations from S. lividans Tr1 (three separate experiments). Therefore, it might be possible that in this last transformant some integrational or recombinational event has taken place between host chromosome and the transforming DNA. Single and double digestions of plasmid DNA from transformants Tr4 and Tr6 with different restriction endonucleases showed that they contained the plasmids named pFM4 and pFM6 (Fig. 1). These have 2.1 and 19.6 kb insertions of S. hygroscopicus DNA respectively. We assumed that both carried an identical copy of an HYG gene.

A partial restriction map of pFM4 is presented in Fig. 1 showing a unique restriction site for the enzymes EcoRI and BamHI. This finding was very useful because it allowed us to perform experiments of insertional inactivation. Since

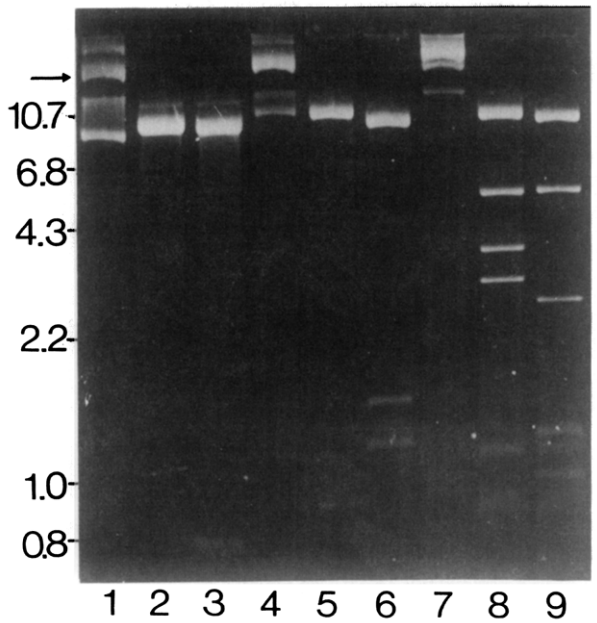

Fig 2.- Agarose gel electrophoretic patterns of intact and restriction endonuclease cleaved DNA from pIJ41, pFM4 and pFM6. DNA was obtained by the mini-prep method (16). The sizes are given in kilobase pairs (kb) and are taken from standard linear DNAs and therefore they can not serve for reference for uncleaved DNA. The weak DNA bands present in the pIJ41 digests correspond to contaminating chromosomal DNA. The arrow refers to chromosomal DNA. pIJ41: 1) uncleaved, 2) +BamHI, 3) +BamHI+PstI. pFM4: 4) uncleaved, 5) +BamHI, 6) +BamHI+PstI. pFM6: 7) uncleaved, 8) +BamHI, 9) +BamHI+ PstI.

no PstI restriction site was present in the S. hygroscopicus DNA, we could recover the whole insert in a 3.1 kb PstI fragment flanked by small streches of pIJ41 DNA (Fig. 2). This fragment was then inserted in the unique PstI site of pIJ350 (Ref. 12 and Fig. 1) and the ligation mixture was used to transform S. lividans 1326. After selection on hygromycin B and thiostrepton containing plates, transformants carrying either plasmid pMZ11.1 (Fig. 1) or pMZ11.2 were isolated. These two plasmids differ only in the orientation of the 3.1 kb PstI fragment. Since the insertion of DNA in the EcoRI site blocks the expression of the HPH activity (M. Zalacaín, umpublished results) plasmid pMZ11.1 or pMZ11.2 may serve as cloning vectors in Streptomyces with one convenient EcoRI site allowing insertional inactivation. In addition the small SstI fragment from pMZ11.1 and pMZ11.2 is dispensable which would leave a single SstI cloning site.

Most yeast, plant and animal cells, are sensitive to hygromycin B. Availability of genetic determinants for phosphotransferases which inactivate hygromycin B could, therefore, allow the construction of cloning vectors for eukaryotic cells containing dominat selective determinants (18,19). Indeed the hygromycin B phosphotransferase genes from two independently isolated bacterial R factors have been fused to Saccharomyces cerevisiae cloning vectors which can use selection to hygromycin B resistance (10,11). The sequence of the HYG gene from E. coli plasmid JR225 has been determined (10) and it will be of interest to compare it with the sequence of the HYG gene from S. hygroscopicus described in this work, which is being determined presently. Both enzymes O-phosphorylate hygromycin B in the 4' position of the deoxystreptamine moiety and have similar molecular weights, 38000 for the pJR225 enzyme, as deduced from its DNA sequence, and 42000 for the S. hygroscopicus enzyme, as deduce by gel filtration (Ref. 10 and J.M. Pardo, umpublished results). These similarities could reflect DNA and protein sequence homology that would throw light on a possible common origin of these antibiotic-resistance genetic determinants from the antibiotic-producing organisms. Actually, DNA sequences of the neomycin phosphotransferase genes from Tn 5, Tn 903, Staphylococcus aureus and Streptomyces fradiae show about 50% homology to one another. The homology is even greater when amino acid sequences are compared (20). These results led Thompson and Gray (20) to propose that these four genes are derived from a common ancestor.

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REFERENCES

1. Cabañas, M., Vázquez, D. and Modolell, J. (1978) Eur. J. Biochem. 87, 21-27.
2. González, A., Jiménez, A., Vázquez, D., Davies, J. and Schindler, D. (1978) Biochim. Biophys. Acta 521, 459-469.
3. Singh, A., Ursic, D. and Davies, J. (1979) Nature 277, 146-148.
4. Mann, R. and Bromer, M. (1958) J. Amer. Chem. Soc. 80, 2714-2716.
5. Leboul, J. and Davies, J. (1982) J. Antibiot. 35, 527-528.

6. Malpartida, F. and Jiménez, A. (1982) Abstr. Int. Symp. Gen. Ind. Microorg. p-1.3, p84.
7. Hopwood, D.A. and Chater, K.F. (1982) Genetic Engineering IV, 119-145. Plenum Publishing Corp. New York & London.
8. Thomson, C.J. Skinner, R.H., Thompson, J., Ward, J.M, Hopwood, D.A. and Cundliffe, E. (1982) J. Bacteriol. 151, 678-685.
9. Pérez-González, J.A., Vara, J. and Jiménez, A. (1983) Biochim. Biophys. Res. Comm. 113, 772-777.
10. Gritz, L. and Davies, J. (1983) Gene, in press.
11. Rao, R.N., Allen, N.E., Hobbs, J.N., Jr., Alborn, W.E., Jr., Jr., Kirst, H.A. and Paschal, J.W. (1983) Antimicrob. Agents Chemother, in press.
12. Chater, K.F., Hopwood, D.A., Kieser, T. and Thompson, C.J. (1982) Curr. Topics Microbiol. and Inmunol. 96, 69-95.
13. Thompson, C.J., Ward, J.M. and Hopwood, D.A. (1980) Nature 286, 525-527.
14. Kieser, T. Hopwood, D.A. Wright, H.M. and Thompson, C.J. (1982) Mol. Gen. Genet. 185, 223-238.
15. Thompson, C.J., Kieser, T., Ward, J.H. and Hopwood, D.A. (1982) Gene 20, 51-62.
16. Bibb, M.J. and Cohen, S.M. (1982) Mol. Gen. Genet. 187, 265-277.
17. Haas, M.J. and Downing, J.E. (1975) Meth. Enzym. 43, 611-627.
18. Jiménez, A. and Davies, J.E. (1980) Nature 287, 869-871.
19. Colbère-Garapin, F. Horodniceau, F., Kourilsky, P. and Garapin, A. (1981) J. Mol. Biol. 150, 1-14.
20. Thompson, C.J. and Gray, G.S. (1983) Proc. Nat. Acad. Sci. (USA), in press.