0273-2289/93/3940—0445$03.00

Gene Manipulation by Protoplast Fusion and Penicillin Production by *Penicillium chrysogenum*

M. K. TAHOUN

Department of Agricultural Industries, Faculty of Agriculture, University of Alexandria, Egypt

ABSTRACT

Hybrids have been obtained by protoplast fusion of nitrate-nonutilizing *cnx*– and acetate-nonutilizing *fac*– mutants of *Penicillium chrysogenum* strains 4/95 and 26/818, respectively. Induced haplodization of the hybrids allowed the recovery of stable segregants, which were screened for penicillin production. The penicillin-producing segregants showed a wide range of titers that reached for a certain mutant to 290–390% increase above the parent strains.

Index Entries: *P. chrysogenum*, hybridization; segregation; penicillin yields.

INTRODUCTION

Penicillin and cephalosporins are β-lactam antibiotics used extensively worldwide to combat infections caused by a diverse array of bacteria. *Penicillium chrysogenum* is used in the manufacture of all clinical penicillins and most oral cephalosporins. In spite of the great interest in this microorganism, the regulatory mechanism that controls gene expression during penicillin biosynthesis is poorly understood (*1,2*). Although the biosynthetic pathway of penicillin is known to be controlled by at least five genes (*3*), the exact location of the genetic system controlling penicillin production has not yet been determined (*4–6*). Therefore, recombinants of high-penicillin-producing strains of *P. chrysogenum* can be produced by the ex-

change of whole chromosomes between the parental strains by means of the parasexual cycle (*7*) or by protoplast fusion (*8,9*). Auxotrophic markers, which are used to select for heterokaryons, result in a decrease in penicillin production. Therefore, the author looked for nitrate-nonutilizing mutants among chlorate-resistant isolates (*10*) and for acetate-nonutilizing mutants amongst fluoroacetate-resistant mutants (*11*).

MATERIALS AND METHODS

Strains

The parental strains used in the present study, *Penicillium chrysogenum* 4/95 and 26/818 with high activity in penicillin production, were provided by the Department of Botany, University of Nottingham, Nottingham, UK. Cultures in regular use were maintained on complete medium and kept at 4°C.

Nitrate-Nonutilizing Mutants

Chlorate resistance in *Penicillium chrysogenum* may result in the inability of the mutants to utilize nitrate as a source of nitrogen. Birkett and Rowlands (*10*) attributed this phonomenon to a loss of nitrate-reductase activity. The medium used for the isolation of chlorate-resistant mutants of *P. chrysogenum* 4/95 is a modified Czapeek-Dox basal medium (*12*) with added NaCl instead of $NaNO_3$. The medium contained (g/L): NaCl, 3.0; KCl, 0.5; $FeSO_4 \cdot 7H_20$, 0.01; KH_2PO_4, 4.0; $MgSO_4 \cdot 7H_2O$, 0.5; Glucose, 40; $NaClO_3$, 94 m*M* and 6 m*M* of L-arginine. The pH was adjusted to 7.0, and agar (2%) was added for solidification and sterilized at 110°C for 30 min.

The chlorate-resistant mutants were replicated on Roberts medium (*13*), which in principle is the basal medium with added nitrogen sources: $NaNO_3$ 12 m*M*; $NaNO_2$, 1.5 m*M*; $(NH_4)_2SO_4$, 0.8 m*M*; adenine hydrochloride, 0.6 m*M*; L-arginine, 6 m*M*; and uric acid, 0.6 m*M*. The mutants were grouped into four classes designated as *nia, cnx, nir,* and CRUN using growth responses on basal medium containing 12 m*M* $NaNO_3$, 1.5 m*M* $NaNO_2$, or 0.6 m*M* adenine hydrochloride at pH 7.0.

Fluoroacetate-Resistant Mutants

Fluoroacetate-resistant mutants are mutants that cannot utilize acetate as a carbon source (fac^-) according to Aprion (*11*). The medium used for that purpose contained (g/L): Na-succinate, 10.0; KH_2PO_4, 3.0; $NaNO_3$, 2.0; KCl, 1.0; and $MgSO_4 \cdot 7H_2O$, 0.5. The pH was adjusted to 6.1, agar was added in 2%, and sterilization was carried out at 130°C for 30 min. *Penicillium chrysogenum* 26/818 (1.2×10^7 spores/mL of a spore

suspension) was laid over the above medium and incubated at 28°C for 40 h, followed by an overlaying of 5 mL of the medium with added fluoroacetic acid at concentrations from 0.1 to 1.0%. The plates were further incubated until the appearance of fluoroacetic-acid-resistant colonies, which were further identified with respect to their inability to utilize acetate as a carbon source. The medium used for the assessment of acetate nonutilization is that as recommended previously (*11*) and contained (g/L): ammonium acetate, 12.0; NaCl, 2.0; KH_2PO_4, 3.0; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.001; and $ZnSO_4 \cdot 7H_2O$, 0.0088.

Media

The complete medium (CM) contained (g/L): yeast extract (Oxoid), 2.5; malt extract (Oxoid), 5.0; D-glucose 10.0; agar, 20.0; $CuSO_4 \cdot 5H_2O$, 0.001, whereas the minimal medium (MM) was used for phenotypic characterization of segregants (*14*). Media used to produce mycelium for protoplast isolation and regeneration, and for hybrid propagation were described previously (*15,16*). The fermentation medium (FM) for penicillin production was that of Macdonald et al. (*12*), except that the precursor was phenylacetic acid (Sigma, 0.2 g/L). The medium used for the biologicol assay of penicillin was nutrient agar (Oxoid). Fermentation agar medium (FAM) was FM solidified with 3% agar, but without the addition of white mineral oil or soya bean oil.

Protoplast Isolation and Fusion

Protoplasts were prepared using a mixture of Novozym 234 and Cellulase CP as described for *Aspergillus* species (*16*). The procedures used for protoplast fusion and hybrid isolation were as described previously (*17,18*). Stable segregants, suggested to be haploid, were obtained by growing the hybrids on CM containing benlate at a concentration of 0.5 μg/mL and supplemented with the nutritional requirements of the parents. The segregants were checked for stability on fresh medium containing benlate and subsequently phenotypically characterized by replication onto appropriately supplemented media.

Agar Disk Assay

The ability of segregants to produce penicillin was determined using the screening method described by Ditchburn et al. (*19*). Each of the tested strains was inoculated onto two disks each 9 mm of FAM and incubated at 28°C for 3 d in an atmosphere of 100% relative humidity. Disks were transferred to assay plates prepared as described for quantitative determination of penicillin. Standards incorporated on each assay plate were prepared by adding penicillin to 20 mL of FAM. Agar disks containing penicillin were then removed and placed on the assay plates.

Methods for Shake Flask Culture

Strains were grown on CM slants for 7 d at 28°C. The spores from each slant were suspended in 5 mL distilled water, tipped into a 250-mL Erlenmeyer flask containing 32 mL FM, and incubated at 28°C for 3 d on a rotary shaker (200 rpm). Fermentation broths were harvested by centrifugation, and the assays were carried out on (12×12 in) assay plates. Two layers of nutrient agar were poured onto the leveled plate; the top layer was seeded with *Bacillus subtilis* NCTC 8236, which was sensitive to penicillin.

Disks of agar were removed, which were punched with a sterile cork borer (9 mm i d) in a 6×6 arrangement of the 36 wells on each plate. Nine were used for standard penicillin G solutions at concentrations 0.5, 2, and 4 U/20 μL. The remaining wells were used for the unknown samples. Twenty microliters of the test and standard solutions were added to each well, and the assay plates were incubated overnight at 28°C. The diameter of zones was measured using calipers and converted to penicillin units by reference to regression equations calculated from penicillin standards ($Y = 11.97 + 1.53\,X$, where Y is the diameter of the clearance and X is penicillin G *U*/20 μL). Each strain was grown in three replicate fermentation flasks; each replicate was assigned to a single well in a different assay plate. All assays were done in duplicate.

RESULTS AND DISCUSSION

The identification of the fluoroacetate-resistant mutants was carried out in two steps. In the first step, fluoroacetate-resistant mutants were isolated by growing *Penicillium chrysogenum* 26/818 at high density (1.2×10^7 spores/mL of a spore suspension) on succinate agar medium in the presence of fluoroacetic acid at concentrations between 0.1 and 1%. The frequency of the resistant mutants varied between 1×10^{-6} and 9.5×10^{-7} colonies at 0.1 and 1% of fluoroacetic acid, respectively. Further identification was performed by growing the resistant mutants on acetate medium. Accordingly, it could be possible to differentiate the acetate-nonutilizing mutants *fac*$^-$ from fluoroacetate-resistant, but acetate-utilizing *fan* (revertents). Out of 71 fluoroacetate-resistant mutants, only seven mutants could not utilize acetate as the only carbon source (*fac*$^-$). These were used for subsequent investigations concerning hybridization. The chlorate-resistant mutants of *P. chrysogenum* 4/95 were isolated by growing a heavy spore suspension of 4.8×10^7 spores/mL on basal medium containing ClO_3^-. The mean frequency value of chlorate-resistant mutants was 2.27×10^{-6}. Further investigation for classifying the chlorate-resistant mutants was carried out on different nitrogen sources. The mutant classes reported previously (*20,21*) were as follows:

Table 1
Distribution of Chlorate-Resistant Mutant Types of *P. chrysogenum* on Different Nitrogen Sources Including Nitrate, Nitrite, and Adenine[a]

Mutant type	No. of colonies	Mutant distribution, %
cnx	67	42
nia	243	27
nir	11	7
CRUN	39	24

[a]Total no. of tested chlorate resistant mutants was 160 colonies.

Table 2
Penicillin Yields U/mL in Shake Flask Cultures by *P. chrysogenum*, *cnx*$^-$, and *fac*$^-$ Mutants[a]

Strain	Penicillin U/mL
P. chrysogenum	
4/95	84
26/818	73
Mutants	
cnx$^-$	51
fac$^-$	41

[a]Mean of 7 replicates each.

1. *nia* mutants: These showed very sparse growth on nitrate;
2. *cnx* mutants: These showed very sparse growth on nitrate and much reduced growth on adenine.
3. *nir* mutants: These showed more growth on nitrate than *nia* and *cnx* mutants, but less than wild-type; and
4. *nii nia* mutants: These were relatively rare and could not be identified among the isolated chlorate-resistant mutants.

The distribution of mutant types isolated on nitrate, nitrite, or adenine is shown in Table 1. The data indicate that *cnx* and *nia* mutants represented about 70% of total chlorate-resistant mutants, whereas the percentage of *nir* mutants was the lowest among mutants identified, about 7%. On the other hand, CRUN mutants that were resistant to chlorate and able to utilize nitrate as sole nitrogen source (*20*) represented about 24% of the total mutants. There was a complete lack of CRUN mutants previously classified by Birkett and Rowlands (*22*).

The influence of converting *P. chrysogenum* 26/818 and 4/95 into acetate- (*fac*$^-$) and nitrate-nonutilizing (*cnx*$^-$) mutants was accompanied by a decrease in penicillin yields as shown in Table 2. That finding contradicts previously unpublished results (*8*), which indicated that the inability

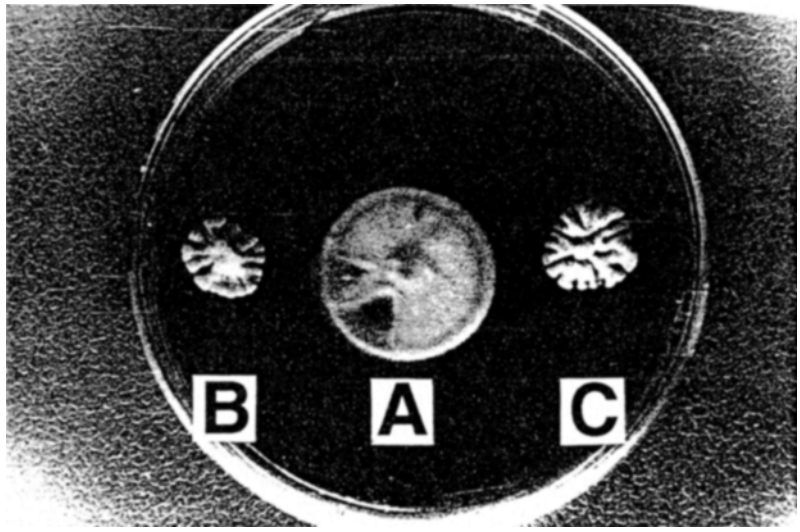

Fig. 1. Induced haplodization and sector formation of the hybrid obtained by protoplast fusion (A) of *fac*$^-$ (B) and *cnx*$^-$ (C) mutants of *P. chrysogenum*.

of *P. chrysogenum* to use nitrate as a nitrogen source and acetate as a carbon source was used as a genetic marker in crosses between high-*Penicillium* strains without affecting their production capacity during fermentation.

About 10^7 protoplasts each of *cnx*$^-$ and *fac*$^-$ mutants were mixed, and fusion was induced by 30% PEG 4000. Balanced heterokaryons could be detected by the complementation of auxotrophic markers on MM containing acetate and nitrate as carbon and nitrogen sources, respectively. Heterokaryons were selected from the plates and plated on MM from which heterozygous diploids were isolated as prototrophic colonies. These colonies were confirmed as diploids by their larger conidial diameter, compared to the haploid parent, by their instability on benlate, and by the isolation of recombinant segregants after haplodization. Haploid segregants were obtained by inoculating diploid conidia onto CM containing benlate. After 7 d of incubation, sectors characterizing segregation were identified (Fig. 1). Segregants were purified, and their ploidy determined by a comparison of their conidial diameter with those of the diploid and the haploid parents, and by their stability on benlate at a concentration of 0.5–1.0 μg/mL. Forty-nine colonies were selected from 1000. These were inoculated in duplicate on agar disks of FAM and incubated at 28°C for 3 d. Disks were transferred to assay plates prepared for quantitative determination of penicillin with standard amounts of penicillin solution included on each bioassay plate.

Out of 49 segregants tested (Fig. 2), 45 produced more penicillin than the *cnx*$^-$ mutant, whereas all segregants revealed higher penicillin titers

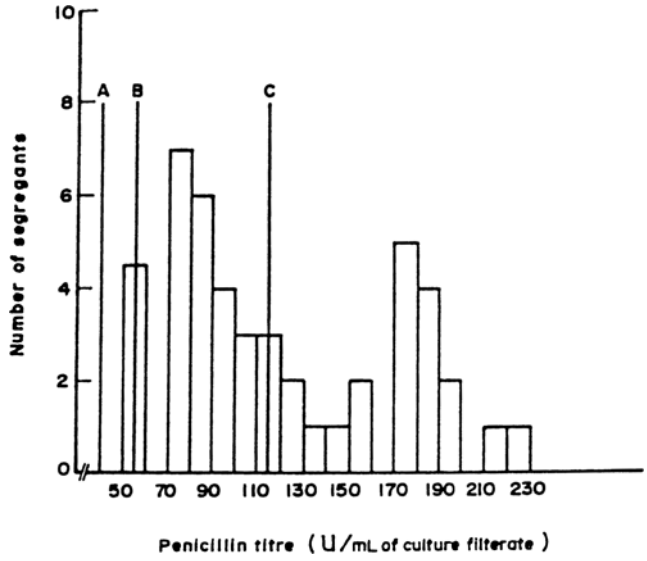

Fig. 2. (A), Titer of *fac*$^-$ parent; (B), titer of *cnx*$^-$ parent; (C), mean of progeny.

than the *fac*$^-$ mutant. The *fac*$^-$, *cnx*$^-$ mutants and 19 segregants that showed high penicillin yields were each tested in shake flask cultures. The penicillin-producing segregants displayed a wide range of titers as shown in Table 3. Out of the tested segregants, 16 revealed higher penicillin yields than the *cnx*$^-$ parent, whereas all segregants showed higher penicillin titers than the *fac*$^-$ mutant. Among segregants investigated, segregant 19 exhibited the highest increase in penicillin yield, 290 and 390% higher than the two parents *cnx*$^-$ and *fac*$^-$ used in the hybridization. Such increase in yields is much greater than that reported previously, 5–125%, either by direct transformation of modified genes (5) or by protoplast fusion of high-producing strains of *P. chrysogenum* (*8,9*).

The mean penicillin titer of the 19 segregants (107 U/mL) was significantly higher than that of *fac*$^-$ and *cnx*$^-$ mutants ($t_{18}=5.64$ for *fac*$^-$ and 4.74 for *cnx*$^-$, $p=0.05$ level). An analysis of variance, Table 4 indicates that the difference in titers between the tested segregants was significant. For comparisons of penicillin yields of the 19 segregants, the least significant difference (LSD) was calculated and compared with the difference in penicillin yields between segregants and *cnx*$^-$ or *fac*$^-$ mutants. Eleven segregants showed more significant penicillin yields than the *fac*$^-$ mutant, whereas 10 segregant displayed more significant penicillin titers than the *cnx*$^-$ mutant (Table 3).

The results presented here suggest that intra-species hybridization, mediated by protoplast fusion, can be instrumental in the generation of strains with increased antibiotic production. Moreover, since many in-

Table 3
Segregants with Improved Penicillin Titer[a]

Strain	Penicillin yield U/mL		
	Segregant, (X)[b]	Difference between	
		Segregants and *fac*$^-$	Segregants and *cnx*$^-$
Mutant			
fac$^-$	41		
cnx$^-$	51		
Segregants			
S1	43	2	
S2	43	3	
S3	48	7	
S4	69	28	18
S5	70	29	18
S6	72	31	20
S7	72	31	21
S8	74	33	23
S9	86	45*	34
S10	99	59*	48*
S11	102	61*	51*
S12	104	63	53*
S13	112	71*	61*
S14	150	109*	99*
S15	157	116*	105*
S16	168	127*	116*
S17	179	139*	128*
S18	186	145*	135*
S19	201	161*	150*

[a]Least significance difference (LSD)=35.
[b]X=Mean penicillin titer.

dustrial important fungi are imperfect (e.g., *Penicillium chrysogenum, Aspergillus niger, Cephalosporium acremonium*), the technique of parasexual recombination via protoplast fusion has important commercial potential.

ACKNOWLEDGMENTS

The research was supported partially by a British Council Scholarship in the UK. I thank Professor J. F. Peberdy of the Department of Botany, the University of Nottingham for kind help and Dr. F. El-Naklawy of the University of Alexandria for help in the statistical analysis.

Table 4
Analysis of Variance of Penicillin Titer (U/mL)
Among 19 Segregants Grown in Shake Flask Cultures

Source of variance	Degree of freedom	Sum of squares	Mean square	Variance
Treatment	22	164827.48	7492.15	16.39[a]
Error	46	21026.45	457.09	
Total	68			

[a] Statistically significant at $p = 0.05$ level.

REFERENCES

1. Demain, A. L. (1983), in *Antibiotics Containing the β-lactam structure*, Demain, I. A. L. and Solmon, N. A., eds., Springer-Verlag International, New York.
2. Martin, J. F. and Liras, P. (1985), *Trends Biotechnol.* **3,** 39.
3. Normansell, P. J. M., Normansell, I. D., and Holt, G. (1979), *J. Gen. Microbiol.* **112,** 113.
4. Cantoral, J. M., Diez, B., Barredo, J. L., Alvarez, E., and Martin, J. F. (1987), *Biotechnology* **5,** 494.
5. Veenstra, A. E., Van Solingen, P., Bovenberg, R. A. L., and Van der Voort, L. H. M. (1991), *J. Biotechnol.* **17,** 81.
6. Bull, J. H., Smith, D. J., and Turner, G. (1989), *Eur. Pat. Appl. EP 311,* 273.
7. Pontecorvo, G. and Sermonti, C. (1953), *Nature* **172,** 126.
8. Van der Beek, C. P., Braat, P. J., and Kerkhof-Tersmette, W. G. (1987), Unpublished data. Gist-brocades N-V., Research Dept. of Yeast and Fungal Genetics. P. O. Box 1, 2600 Ma Delft. The Netherlands.
9. Miu, I., Bugiu, E., Mustea, A., Oroian, M., and Sauciuc, A. (1988), *Rev. Chim. (Bucharest)* **39,** 766.
10. Birkett, J. A. and Rowlands, R. T. (1981), *J. Gen. Microbiol.* **123,** 281.
11. Apirion, D. (1965), *Genet. Res.* **6,** 317.
12. Macdonald, K. D., Hutchinson, J. M., and Gillet, W. A. (1963), *J. Gen. Microbiol.* **33,** 365.
13. Robey, C. F. (1959), *J. Gen. Microbiol.* **20,** 540.
14. Pontecorvo, G., Roper, J. A., Hemmons, L. M., Macdonald, K. D., and Bulton, A. W. J. (1953), *Adv. Genet.* **5,** 141.
15. Kevei, F. and Peberdy, J. F. (1977), *J. Gen. Microbiol.* **102,** 255.
16. Hamyln, P. F., Bradshaw, R. E., Mellon, F. M., Santiago, C. M., Wilson, J. M., and Peberdy, J. F. (1981), *Enzyme Microb. Technol.* **3,** 321.
17. Anne, J. and Peberdy, J. F. (1976), *J. Gen. Microbiol* **92,** 413.
18. Kevei, F. and Peberdy, J. F. (1979), *Mol. Gen. Genet.* **170,** 213.
19. Ditchburn, P., Giddings, B., and Macdonald, K. D. (1974), *J. Appl. Bacteriol.* **37,** 515.
20. Cove, D. J. (1976), *Heredity* **36,** 191.
21. Cove, D. J. (1976), *Mol. Gen. Genet.* **146,** 147.
22. Birkett, J. A. and Rowlands, R. T. (1981), *J. Gen. Microbiol.* **123,** 281.