Biochemical Properties of Haploid and Diploid Strains of *Penicillium chrysogenum*

S. G. PATHAK AND R. P. ELANDER

*Wyeth Laboratories, Inc., Antibiotic Division, West Chester, Pennsylvania 19380*

Received for publication 1 June 1971

An intensive parasexual genetics program in which industrial strains of *Penicillium chrysogenum* were used culminated in the isolation of a number of heterozygous diploid strains. The diploid clones were selected from heterokaryons formed from matings between mutant strains having complementary biochemical and conidial color markers. Several diploid cultures were compared with their haploid wild-type parents and other distantly related production strains on the basis of a variety of cultural and physiological criteria. The diploid strains characteristically produced conidia of larger volume and higher deoxyribonucleic acid content. Some were vigorous with respect to growth rate and onset and degree of conidiation. One diploid strain (WC-9) had a 46% greater oxygen uptake rate and oxidized glucose at a 57% greater rate than its haploid parent (M-2). It also produced 33% higher concentrations of β-galactosidase, 66% more alkaline protease, and 53% more glucose oxidase than the M-2 haploid parent. The selection of rare stable diploid mold cultures through the use of parasexual genetics offers a unique approach to the direct selection of mutants with potential for increased enzyme formation.

In addition to the highly successful techniques based on conventional methods of mutation and selection, industrial strain-development laboratories have utilized parasexual genetic techniques for the development of potent, high-producing, industrially important fungi. Although Pontecorvo and Roper (13) and Pontecorvo and Sermonti (14) elucidated the details of this novel genetic process in fungi and indicated its potential for developing improved industrial strains, later investigations by numerous workers were disappointing with respect to its industrial application. Elander (5, 6) reported on an intensive large-scale industrial strain development program with *Penicillium* and described a stable heterozygous diploid strain which synthesized high concentrations of penicillin. A recent report described the production of benzylpenicillin and penoxymethylpenicillin by the haploid and diploid strains of *P. chrysogenum* described herein [R. P. Elander et al., Genetics of industrial microorganisms (Prague), in press]. This report summarizes certain biochemical properties of representative haploid and diploid production strains of *P. chrysogenum*.

**MATERIALS AND METHODS**

**Strains.** Strains of *P. chrysogenum* were maintained in lyophilized ampoules to obviate back-mutation and degeneration with respect to penicillin formation. The M designation refers to actual production usage. The production strains are characterized by profuse green (wild-type) conidiation on slant culture and are prototrophic with respect to their nutritional requirements. Parasexual recombination was observed in heterokaryotic cultures synthesized from complementary auxotrophic mutants derived from the wild-type commercial strains designated M-2 and M-30. The lineage of several mutants and recombinants derived therefrom is summarized in Fig. 1.

**Matagens.** The source of ultraviolet (UV) radiation (253 nm) was a low-pressure mercury arc lamp, model PCQ-X1, obtained from UltraViolet Products, Inc., San Gabriel, Calif. A dose rate of 300 to 400 μW cm⁻² sec⁻¹ was used for all UV-radiation experiments. The intensity measurements were determined with the aid of a UV intensity meter obtained from UltraViolet Products, Inc.

With nitrogen mustard, the procedure of Stahmann and Stauffer (17) was followed for induction of mutation. Methyl-bis-(β-chloroethyl)-amine (obtained as mechlorethamine hydrochloride from Aldrich Chemical Co., Milwaukee, Wis.) was used.

*N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) was obtained from Aldrich Chemical Co. The routine technique used a concentration of 0.025 M NTG and an exposure of the spores for periods ranging from 0 to 20 min. This mutation protocol was adapted from those used by Adelberg et al. (1) and Moore (9).

With nitrous acid, the procedure described by Siddiqi (16) was used. The conidia of *P. chrysogenum*...
were exposed to a concentration of 0.017 M nitrous acid in tris(hydroxymethyl)aminomethane (Tris) buffer for a period ranging from 0 to 30 min.

Glucose oxidation. The broths were filtered through Whatman no. 1 paper in a glass funnel. The filtrate was diluted 1:200, and the residual glucose was analyzed by the method of Sumner (18).

Spore germination. Washed conidia were diluted to approximately 10^8 spores/ml and inoculated as suspensions into Erlenmeyer flasks (250 ml) containing 50 ml of sterile Czapek-Dox solution. The flasks were incubated at 25 C on a New Brunswick rotary shaker (250 rev/min). Samples were removed at 16 hr, and direct counts were made with the aid of a Levy hemocytometer. A total of 200 spores were counted for each strain.

Respiration studies. Standard Warburg manometric methods (19) were used. Potassium hydroxide (0.2 ml of 20%) was added to the center well of all flasks to absorb carbon dioxide. A Gilson model G-8 differential respirometer was employed.

Parasexual techniques. Complete medium consisted of corn steep liquor (Corn Products), 0.5%; Bacto-Peptone (Difco), 0.5%; yeast extract (Difco), 0.5%; Casamino Acids (Sheffield N-Z Case), 0.25%; corn dextrin, 3.0%; FeSO_4·7H_2O, 0.001%; KCl, 0.05%; MgSO_4·7H_2O, 0.05%; KH_2PO_4, 0.1%; NH_4NO_3, 0.3%; and agar (Difco), 2.0%; adjusted to pH 5.5.

Minimal medium contained NH_4NO_3, 0.3%; KH_2PO_4, 0.1%; MgSO_4·7H_2O, 0.05%; KCl, 0.05%; FeSO_4·7H_2O, 0.001%; sucrose (reagent grade), 3.0%; and Ionagar (Colab Laboratories, Inc., North Chicago, Ill.), 1.0%; pH 5.5.

Nomenclature. The system of abbreviations for nutritional, conidial color, and colonial growth mutants (Fig. 1, Table 1) was adopted according to the proposals of Demerec et al. (3) and Sermonti (15).

Isolation of heterozygous diploids and segregant strains. Homogenized spore suspensions of the two complementary biochemically deficient parental strains were inoculated into complete medium broth and incubated at 25 C for 7 to 10 days. The mycelial mass was then removed, washed three to five times with minimal medium, and teased apart on the surface of minimal agar. Diploid conidia were isolated from green diploid sectors in heterokaryotic mycelial fans or from green conidiating colonies derived from spores from heterokaryotic growth which had previously been diluted and plated out on either complete or minimal medium. Segregants were selected as sectors formed in diploid mycelium.

Cultures propagated on complete medium were used as sources of conidia for microscopic measurements. Conidia were examined under high-power magnification (400 times) and measured to the nearest 0.25 µm. Conidial volumes were calculated by the formula V = 1/6 π d^3. Haploid and diploid conidia or mycelia, or both, were also differentiated by deoxyribonucleic acid (DNA) content and enzyme level.

DNA measurements. A concentration of approximately 10^8 spores was obtained from an inoculated 1-liter Erlenmeyer flask containing 75 g of moistened medium-grade cracked corn. The diphenylamine colorimetric procedure described by Ishitani et al. (7) and Arrighi et al. (2) for DNA analysis was used. Highly purified DNA (Nutritional Biochemicals Corp., Cleveland, Ohio) served as standard. Spore densities were determined with the aid of a Levy counting chamber.

Enzyme assays. Mycelia of the various strains were harvested from Erlenmeyer flasks (250 ml) containing a growth medium consisting of corn steep liquor (6%) and lactose (2%). The flasks had been inoculated with

Fig. 1. Lineage of mutant and recombinant strains of Penicillium chrysogenum. Abbreviations: ylo, alb, and fwn designate yellow, hyaline, and tan conidiation; dwf, petite colony; met, ade, arg, ane, and rib designate nutritional requirement for methionine, adenine, arginine, thiamine, and riboflavin, respectively; UV, ultraviolet radiation (253 nm); NA, nitrous acid; NG, N-methyl-N'-nitro-N-nitrosoguanidine.
spores of the various strains and incubated at 25 C on a New Brunswick rotary shaker for 72 hr. Lyophilized mycelium, further desiccated and ground with coarse quartz sand in a mortar and pestle, was suspended in 0.1 M phosphate buffer (pH 6.0) and assayed for β-galactosidase according to the method of Neidhardt and Boyde (11). For determination of alkaline protease, the various strains were propagated on a high-protein medium described by Dworschack et al. (4). The mycelia were harvested from Erlenmeyer flasks (250 ml) containing 50 ml of medium consisting of soybean meal (1.0%), corn meal (2.0%), and CaCO₃ (0.5%). Total protein was determined with the Folin phenol reagent according to the procedure of Lowry et al. (8). Bovine serum albumin was used as the standard. The assays were made on filtered broth, because most of this enzyme is extracellular. Glucose oxidase was determined according to the procedure developed by Underkofler (20). The mycelial extracts were compared with a purified glucose oxidase enzyme standard obtained from Nutritional Biochemicals Corp.

RESULTS

Properties of the heterozygous diploid strains: conidial volumes and DNA content. The spore volume and DNA content of the two parental wild-type strains (M-2 and M-30) and some selected haploid and diploid derivatives are shown in Table 1. The chosen examples represent several different phenotypic combinations of conidial color, nutritional requirement, and biochemical adequacy. The diploid strains WC-9, R-10, and R-18 characteristically exhibited larger spore volume and DNA content than their respective wild-type haploid grandparents (M-30 or M-2) or their complementary biochemically deficient parents. Strain 105, a thiamine-requiring mutant, had an unusually large (76.93 μm³) spore volume and a greater DNA content (1.26 × 10⁻⁷ μg) than the wild-type parental strain (M-30). When the dwarf marker was introduced into the mutant, the conidial volume and DNA content were reduced by nearly one-half.

Spore germination. Table 2 shows the results of a comparative spore germination study between a particularly vigorous diploid strain (WC-9) and two haploid strains (M-2 and M-30). Equivalent numbers of conidia (adjusted to approximately 5 × 10⁷ spores/ml) were obtained from slants of similar age (7 days at 25 C) and inoculated as suspensions into Erlenmeyer flasks (250 ml) containing sterile Czapek-Dox solution (50 ml). The inoculated flasks were then incubated at 25 C on a rotary shaker (250 rev/min). After a period of 16 hr, samples were removed and placed on a Levy hemocytometer for microscopic observation (430 times). The average number of spores counted was 200 for each strain. The diploid WC-9 strain showed nearly 50% germination at 24 hr in the chemically defined medium. In contrast, the two haploid strains showed only 34 and 37% germination.

Respiration and glucose utilization. Strains M-2 and M-30 were compared with the WC-9 diploid strain on the basis of oxygen uptake and glucose oxidation. Washed mycelial suspensions of the three strains were added to standard Warburg flasks containing 0.4% glucose solution. The flasks were then placed on the Gilson differential respirometer apparatus and submerged in a water bath at 25 C. Table 3 shows the average results of three separate experiments with flasks run in duplicate for each strain. The diploid

Table 1. Spore volume and DNA content of selected haploid and diploid strains of Penicillium chrysogenum

| Strain | Genotype* | Ploidy | Spore volume (μm³) | DNA per spore (μg) |
|--------|-----------|--------|-------------------|-------------------|
| M-30   | W⁺        | N      | 54.38             | 0.26 × 10⁻⁷       |
| 41     | arg       | N      | 53.00             | 0.35 × 10⁻⁷       |
| 107    | alb arg   | N      | 27.54             | 0.26 × 10⁻⁷       |
| 115    | fwn rib   | N      | 22.43             | 0.33 × 10⁻⁷       |
| R-18   | alb arg/fwn rib | 2N   | 81.10             | 0.79 × 10⁻⁷       |
| 105    | ane       | N      | 76.93             | 1.26 × 10⁻⁷       |
| 134    | dwf ane   | N      | 36.08             | 0.69 × 10⁻⁷       |
| SY-24  | ylo       | N      | 69.77             | 0.88 × 10⁻⁷       |
| 140    | ylo arg   | N      | 42.1              | 0.66 × 10⁻⁷       |
| R-10   | dwf ane/ylo arg | 2N | 65.5              | 1.15 × 10⁻⁷       |
| M-2    | W⁺        | N      | 34.99             | 0.44 × 10⁻⁷       |
| WC-2   | ylo met   | N      | 36.78             | 0.57 × 10⁻⁷       |
| WC-8   | alb ade   | N      | 29.75             | 0.45 × 10⁻⁷       |
| WC-9   | ylo met/alb ade | 2N | 61.49              | 0.83 × 10⁻⁷       |

* Abbreviations: W⁺, wild-type; ylo, alb, and fwn designate yellow, hyaline, and tan conidiation; dwf, petite colony; met, ade, arg, ane, and rib designate nutritional requirement for methionine, adenine, arginine, thiamine, and riboflavin, respectively.
Table 2. Spore germination in haploid and diploid strains of Penicillium chrysogenum

| Strain designation | Ploidy | Per cent germination in Czapek-Dox solution<sup>a</sup> |
|--------------------|--------|-----------------------------------------------------|
| M-2                | N      | 37.1                                                |
| M-30               | N      | 33.9                                                |
| WC-9               | 2N     | 47.5                                                |

<sup>a</sup> Average of 200 spores; 24-hr incubation at 25 C.

Table 3. Comparison of oxygen uptake and glucose oxidation in haploid and diploid strains of Penicillium chrysogenum

| Strain | Ploidy | $O_2$/hr (liters per mg of mycelium) | Glucose oxidized (mg/ml)<sup>a</sup> |
|--------|--------|--------------------------------------|-------------------------------------|
| M-2    | N      | 5.03                                 | 0.90                                |
| M-30   | N      | 5.06                                 | 1.09                                |
| WC-9   | 2N     | 7.34                                 | 1.41                                |

<sup>a</sup> Glucose supplied: 4 mg per ml per Warburg flask.

Table 4. Comparison of $\beta$-galactosidase and glucose oxidase activity in haploid and diploid strains of Penicillium chrysogenum

| Strain designation | Ploidy | $\beta$-Galactosidase<sup>b</sup> | Glucose oxidase<sup>b</sup> |
|--------------------|--------|---------------------------------|----------------------------|
| M-2                | N      | 80.26                           | $1.275 \times 10^4$        |
| M-30               | N      | 89.68                           | $1.575 \times 10^4$        |
| WC-9               | 2N     | 106.40                          | $27.150 \times 10^3$       |

<sup>b</sup> Expressed as units per hour per milligram of protein.

The culture (WC-9) respired at a rate 46% greater than that of its parent (M-2) and approximately 45% greater than that of the M-30 haploid production strain. At the completion of the experiments, the broths were examined for residual glucose levels. The diploid strain oxidized considerably more glucose than either of the haploid strains: 1.41 mg/ml versus 0.90 mg/ml and 1.09 mg/ml for the M-2 and M-30 cultures, respectively (Table 3).

Enzyme studies. Tables 4 and 5 summarize the comparative mycelial amounts of $\beta$-galactosidase, glucose oxidase, and alkaline protease. The diploid strain (WC-9) possessed 33 and 19% more $\beta$-galactosidase activity, respectively, than the two haploid strains M-2 and M-30 (Table 4). Table 4 summarizes the glucose oxidase data for the three strains. Air-dried ethanol extracts of WC-9 diploid mycelium contained 53 and 24% more glucose oxidase, respectively, than did similar extracts of strains M-2 and M-30. As shown in Table 5, the alkaline protease activity of the diploid WC-9 strain (8.92 units/mg of protein) was considerably higher than the activity of either of the two haploid strains (60% higher than M-2 and 254% higher than M-30).

Table 5. Alkaline protease activity in haploid and diploid strains of Penicillium chrysogenum

| Strain | Ploidy | Alkaline protease |
|--------|--------|-------------------|
|        |        | Total units       | Units/mg of protein |
| M-2    | N      | 700.4             | 5.58                |
| M-30   | N      | 347.2             | 2.52                |
| WC-9   | 2N     | 1341.5            | 8.92                |

The discovery of parasexuality in asexual molds by Pontecorvo and Roper in 1952 provided a means for planned breeding which resulted in the synthesis of vigorous diploid strains with increased capacity to synthesize economically important fermentation products. The technique consists of combining two strains with particularly interesting properties to form a diploid with more desirable properties. For enhanced penicillin production, notable desirable properties include more rapid, early synthesis of secondary metabolites generating penicillin, more abundant conidiation to facilitate long-term preservation, more efficient sugar and precursor utilization, greater synthesis of 6-aminopenicillanic acid, and low levels of penicillin acylase.

The studies described herein on the conidial volume and DNA content of various haploid and diploid strains of Penicillium clearly indicate that strains R-10, R-18, and WC-9 possess higher ploidy levels than their respective wild-type and related auxotrophic parents. The data also point out that considerable variation in spore size and DNA content may occur when major mutants are utilized for genetic recombination studies. It appears that both conidial volume and DNA content should be correlated for proper ploidy determination. The present study also suggests that the diploid strains should be compared with the immediate parents in the lineage rather than with distantly related great-grandparent strains. In the present study, two wild-type strains of differing lineage were utilized. Although the strains have common ancestry in the Wisconsin series, there is considerable variation in conidial size and DNA content (Table 1). The introduction of biochemical markers into strains leads to
auxotrophy and perhaps to other unusual pleiotropic effects. In this study, the introduction of the thiamine marker (ane) into strain 105 resulted in large conidium formation compared with the wild-type parent (strain M-30). This particular auxotrophic mutant had a greater conidial DNA content than any of the diploid strains analyzed thus far in our laboratories. However, the introduction of the dwarf marker (dwl) into the strain (no. 134) reduced the DNA content by nearly one-half.

The diploid strain WC-9 appears to be a particularly vigorous culture compared with the two wild-type haploid production cultures (M-2 and M-30). This culture, when inoculated into fresh agar medium, completes its asexual life cycle (spore to spore) and exhibits abundant green diploid conidiation after 3 to 4 days of incubation. In contrast, the M-2 and M-30 haploid strains generally require an incubation period of 5 to 6 days before new green conidiation is observed. With this background, it is not surprising that the WC-9 culture exhibits more vigor with respect to more rapid spore germination, oxygen uptake, and glucose oxidation.

According to Pardee (12), one method for improving the rate of enzyme synthesis in industrially important microorganisms is to increase the number of structural genes per given cell. When extra copies of the gene are in the cell, the enzyme can usually be produced in proportionately larger amounts. In *Escherichia coli*, cells with increased numbers of structural genes can be selected either by isolating rare mutants possessing several phenocopies, by introducing extra-chromosomal segments (episomes), or by phage-escape synthesis, whereby bacteria can be infected by lysogenic phages which carry structural genes for specific bacterial enzymes in place of phage genetic material.

In filamentous fungi, including those with a predominant yeast phase, parasexuality provides an excellent technique for obtaining cells with increased numbers of structural genes. Diploid phase yeast strains which show increased vigor with respect to ethanol synthesis and starch hydrolysis have been commonly utilized. The Japanese have improved the production of soy sauce and other fermented foods through diploid hybridization of selected strains of the heterothallic haploid yeast *Saccharomyces rouxii*. Wickerham et al. (21) recently described an unusual diploid isolate of *Candida lipolytica* (*Endomycopsis lipolytica*). Haploid strains of this organism were outstanding in ability to produce lipase and extracellular protease. This particular yeast also showed great potential for converting hydrocarbons to foods and animal feeds. The investigators suggested that the economic yields from technological processes employing this organism might be greatly improved by hybridization of selected lines and production of large-celled diploids.

The WC-9 diploid strain of *Penicillium* described in this report has considerable vigor with respect to the formation of β-galactosidase, glucose oxidase, and alkaline protease (Tables 4 and 5). The WC-9 culture is also an efficient producer of penicillin antibiotics [R. P. Elander et al., Genetics of industrial microorganisms (Prague), in press]. It would appear that the larger diploid cells of this clone possess a greater number of structural genes regulating the formation of these three particular enzymes (and perhaps, as well, other enzymes involved in the biosynthesis of penicillins). The diploid state of the strain is undoubtedly responsible for the enhanced enzyme formation.

Asexual filamentous fungi are utilized in many important industrial processes—antibiotic syntheses, steroid hydroxylations and reductions, and prostaglandin conversions. Despite the large volume of literature pertaining to the use of filamentous fungi in bioconversions of important steroid moieties, we know of no published reports concerning the application of artificially induced diploid strains for more efficient steroid conversions. The use of parasexual genetic techniques to generate stable heterozygous diploid strains, or cultures of higher ploidy, appears to be a unique approach to the selection of highly productive cultures of filamentous fungi.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of Vincent Cullen, Edward Ambush, and Robert DePhillips. The diploid cultures were generated by M. A. Espenshade and Vincent Karesky in the Strain Development and Genetics Laboratories.

LITERATURE CITED

1. Adelberg, E. A., M. Mandel, and G. C. C. Chen. 1965. Optimal conditions for mutagenesis by N-methyl-N'nitro-N-nitrosoguanidine in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. 18:788-795.
2. Arrighi, F. E., J. Bergendahl, and M. Mandel. 1968. Isolation and characterization of DNA from fixed cells and tissues. Exp. Cell Res. 58:47-49.
3. Demerec, M., E. A. Adelberg, A. J. Clark, and P. E. Hartman. 1966. A proposal for a uniform nomenclature in bacterial genetics. Genetics 54:61-84.
4. Dworschack, R. G., H. J. Koeppe1, and A. L. Lagoda. 1952. Proteolytic enzymes of microorganisms. Evaluation of proteinases produced by molds of the *Aspergillus flavus-oryzae* group in submerged culture. Arch. Biochem. Biophys. 41:68-69.
5. Elander, R. P. 1967. Enhanced penicillin biosynthesis in mutant and recombinant strains of *Penicillium chrysogenum*, p. 403-423. In Induced mutations and their utilization. Abb. Deut. Akad. Wiss. Berlin.
6. Elander, R. P. 1969. Applications of microbial genetics to
industrial fermentations, p. 89–114. In D. Perlman (ed.), Fermentation advances. Academic Press Inc., New York.

7. Ishitani, C. Y., Y. Ikeda, and S. Sakaguchi. 1956. Hereditary variation and genetic recombination in Koji molds (Aspergillus oryzae and Aspergillus sojae). VI. Genetic recombination in heterozygous diploids. J. Gen. Appl. Microbiol. 2:401–430.

8. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193:265–275.

9. Moore, D. 1969. The mutagenic action of N-methyl-N'-nitro-N-nitrosoguanidine on Coprinus lagopus. J. Gen. Microbiol. 55:121–125.

10. Mori, H., and H. Onishi. 1967. Diploid hybridization in a heterothallic haploid yeast, Saccharomyces rouxii. Appl. Microbiol. 15:928–934.

11. Neidhardt, F. C., and R. F. Boyd. 1965. Cell biology: a laboratory text, p. 90–98. Burgess Publishing Co., Minneapolis.

12. Pardee, A. B. 1969. Enzyme production by bacteria, p. 3–14. In D. Perlman (ed.), Fermentation advances. Academic Press Inc., New York.

13. Pontecorvo, G., and J. A. Roper. 1952. Genetic analysis without sexual reproduction by means of polyploidy in Aspergilus nidulans. J. Gen. Microbiol. 6:vii–viii.

14. Pontecorvo, G., and G. Sermonti. 1953. Recombination without sexual reproduction in Penicillium chrysogenum. Nature (London) 172:126–127.

15. Sermonti, G. 1969. Genetics of antibiotic producing microorganisms. Wiley-Interscience, London.

16. Siddiqi, O. H. 1962. Mutagenic action of nitrous acid on Aspergillus nidulans. Genet. Res. 3:303–310.

17. Stahmann, M. A., and J. F. Stauffer. 1947. Induction of mutants of Penicillium notatum by methyl-bis-(β-chloroethyl)amine. Science 106:35–36.

18. Sumner, J. B. 1925. A more specific reagent for the determination of sugar in urine. J. Biol. Chem. 65:393–395.

19. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1964 Manometric techniques: manual describing methods applicable to the study of tissue metabolism. Burgess Publishing Co., Minneapolis.

20. Underkofler, L. A. 1938. Properties and applications of the fungal enzyme glucose oxidase. Proc. Int. Symp. Enzyme Chem. (Tokyo), p. 486–490.

21. Wickerham, L. J., C. P. Kurtzman, and A. I. Herman. 1970. Sexual reproduction in Candida lipolytica. Science 167:1141.