0273-2289/92/3302—0083$02.60

Obtaining Mutants for Protoplast Fusion of Gibberellin-Forming *Gibberella fujikuroi* Strains

K. BERMUDEZ TORRES, B. BRÜCKNER,* AND B. MEIER

Friedrich Schiller University Jena, Institute of Microbiology, Neugasse 24, 0-6900 Jena, Germany

Received June 25, 1991; Accepted December 23, 1991

ABSTRACT

Auxotrophic, drug-resistant, nitrate-nonutilizing, and albino mutants have been isolated in *Gibberella fujikuroi* following UV mutagenesis. Protoplasts of complementing auxotrophic strains, mutants with resistance markers, or mutants blocked in different steps of the nitrate assimilatory pathway have been fused to form heterokaryons, diploids, or recombinant haploids. The properties of fusant strains, including gibberellic acid productivity, have been examined and compared to parent strains.

Index Entries: *G. fujikuroi;* protoplast fusion; auxotrophic; drug resistant; nitrate-nonutilizing; albino mutants; gibberellic acid.

INTRODUCTION

Protoplast fusion has been demonstrated as an efficient way to improve industrial microorganisms. Many reports have suggested the possibility of breeding filamentous fungi producing secondary metabolites such as *Aspergillus niger* (*1*), *Penicillium chrysogenum* (*2*), and *Cephalosporium acremonium* (*3*).

Gibberella fujikuroi (Saw.) is well known because of its ability to produce some secondary metabolites. The most important of these are the gibberellins (*4,5*). Gibberellin-producing activity has been increased by

*Author to whom all correspondence and reprint requests should be addressed.

mutagenesis and screening (*6–8*). Calam et al. (*9*) described parasexual recombination of gibberellin-producing strains with different levels of production ability by hyphal anastomosis. No reports are known on protoplast fusion of gibberellin producers.

In a previous paper, we reported on the optimization of a method for protoplast formation from mycelia of *G. fujikuroi* (*10*). The first step in establishing an artificial parasexual cycle by protoplast fusion is the production of mutants. Complementing auxotrophies have often been used as genetic markers (*1,11,12*). In this paper, we report the development of a protoplast fusion system for gibberellin-producing strains of *G. fujikuroi*. Different mutants of this species marked by auxotrophic requirements, drug resistance, genetic blocks in nitrate assimilatory, and carotenoid biosynthetic pathways were used. The genetic state and gibberellin-producing activity of fusant strains were analyzed.

MATERIALS AND METHODS

Microorganisms

Gibberella fujikuroi (Saw.) Wr. m 567 was obtained from the Fungal Culture Collection (Weimar, Germany). From this strain, the improved gibberellic acid (GA_3)-producing mutant 3422 had been obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) treatment (Kölblin et al., 1991). Auxotrophic, albino, and drug-resistant mutants were isolated from this parental strain after UV treatment.

Media

Minimal medium (MM) contained (per liter) 30 g sucrose, 3 g $NaNO_3$, 1 g KH_2PO_4, 500 mg $MgSO_4 \cdot 7H_2O$, 500 mg KCl, 10 mg $FeSO_4 \cdot 7H_2O$, 1 mL trace elements solution, and 20 g Difco agar. For embedding of protoplasts, this MM was used as a soft agar containing 5 g/L Difco agar. The trace element solution contained (in 100 mL) 5 g citric acid, 5 g $ZnSO_4 \cdot 7H_2O$, 1 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 250 mg $CuSO_4 \cdot 5H_2O$, 50 mg $MnSO_4 \cdot H_2O$, 50 mg H_3BO_3, and 50 mg $NaMoO_4 \cdot 2H_2O$. Complete medium (CM) was a 1:2 diluted malt agar. In the protoplast fusion experiments, MM and CM containing 0.6*M* KCl were used as hypertonic media.

For microconidia production, the fungus was incubated on KCl agar (8 g/L KCl, 25 g/L Difco agar). Mycelium for protoplast isolation was produced as described by Brückner et al. (*10*). GA_3 production of fusant strains was carried out in a complete medium described by Darken et al. (*13*).

Mutagenesis

Gibberella fujikuroi was incubated on KCl agar at 28°C in the dark, and microconidia were harvested after 14 d. Suspensions of 10^6 microconidia

per milliliter in distilled water were exposed to a dose of UV light (254 nm), which yielded a 15–30% survival rate.

Isolation of Mutants

Auxotrophic Mutants

After UV irradiation, the auxotrophic mutants were isolated by filtration enrichment (*14*).

Nitrate Assimilation Mutants

Irradiated conidia were plated onto MM with $(NH_4)_2SO_4$ (2 g/L) as sole nitrogen source supplemented with 500 mM $KClO_3$, and plates were incubated for 7–10 d in the dark. Growth of wild-type strains is restricted on chlorate, presumably because chlorate is reduced by nitrate reductase to highly toxic chlorite. Nitrate-nonutilizing mutants are unable to reduce chlorate to chlorite and hence are chlorate-resistant. These so-called *nit* mutants were assigned to different phenotypic classes on the basis of their growth on MM containing one of five different nitrogen sources: $NaNO_3$ (basal MM as described above), $NaNO_2$ (0.5 g/L), hypoxanthine (0.2 g/L), $(NH_4)_2SO_4$ (1 g/L), or uric acid (0.2 g/L). The phenotypes of the *nit* mutants were determined by growth test on these media as described by Correll et al. (*15*).

Albino Mutants

Pigmentless albino strains were isolated from a deep orange morphological mutant AO6. The orange-pigmented strain has the clear advantage in comparison to the white wild-type strain that albino mutants are immediately scorable by visual inspection. Irradiated conidia of the orange strain AO6 were plated on CM, and after 7 d, the plates were scanned for white mutants. Monoconidial clones of each albino mutant were recovered to increase stability of the genetic blocks.

Drug-Resistant Mutants

For isolation of nystatin- and cerulenin-resistant mutants, first the minimum inhibitory concentrations (MIC) for the drugs were determined. They are 40 μg/mL for nystatin and 10 μg/mL for cerulenin. Resistant mutants were isolated after UV irradiation of microconidia on MM supplemented with nystatin and cerulenin at two times their MIC.

Protoplast Production and Fusion

Protoplasts were isolated as described (*10*). Next, 10^7 protoplasts of each of the complementary strains were mixed together and centrifuged for 10 min at 3000 rpm. The pellet was resuspended in 0.5 mL of protoplast fusion medium—an aqueous solution of 25% polyethylene glycol 6000 (Sigma) supplemented with 0.01*M* $CaCl_2$ in 0.01*M* Tris-HCl buffer (pH 7.5) for 15 min at room temperature. Aliquots of 0.1 mL of the sus-

pension were embedded in 9.9 mL MM melted soft agar and plated onto stabilized CM or MM. For the determination of the regeneration rate, unfused protoplasts of each strain were diluted in melted soft agar (10^{-1}–10^{-5}) and poured onto stabilized CM.

Heterodiploid Formation from Fusants (Heterokaryons)

Heterodiploids were induced from a fusant by d-camphor treatment according to Kirimura et al. (*1*). After a third cultivation on MM supplemented with 0.5 g/L d-camphor, microconidia were recovered from the fusant strains, and monospore cultivation was carried out on MM and CM at 28°C. The number of conidia growing on MM was compared with that on CM.

Segregation of Diploids

The ploidy of the colonies that appeared on MM was checked by their stability on CM containing 3.5 μM methylbenzimidazol-2-yl-carbamat (MBC) as haploidising agent (*16*). Following growth on MBC-medium, in some cases, faster-growing or pigmented sectors were segregated from the central areas of diploid mycelia. After 14 d of cultivation at 28°C, the segregants were checked for morphological stability on fresh CM containing MBC and purified by single conidia colonies.

Gibberellic Acid Production

GA_3 production by shaking culture was carried out in 50-mL shaken flasks containing 20 mL of Darken medium (*13*). The cultures were incubated for 7 d on a rotary shaker (240 rpm) at 28°C. GA_3 was determined spectrofluorimetrically by measurement of the fluorescence of the GA_3-sulfuric acid-complex (excitation at 310 nm, emission at 400 nm) on a Carl Zeiss spectrophotometer with fluorescence detector.

RESULTS

Mutants

The first step in preparing for fusion experiments was the production of different marked mutants of a high-producing parental strain of *G. fujikuroi* following UV irradiation of microconidia. Auxotrophic mutants are often decreased in their ability to produce secondary metabolites (*17*). We obtained 16 different auxotrophic strains, from which only three (requiring glutamine, tryptophane, or threonine) have the same or slightly higher productivity as the wild-type strain (Table 1). Therefore, in addition to auxotrophs, we also isolated other types of marked mutants.

Mutants blocked in the nitrate assimilatory pathway were easy to isolate on selection plates containing $KClO_3$. Phenotypes consistent with mutations in the structural gene for nitrate reductase (*nit* 1) and in the

Table 1
Mutants Produced from Microconidia of *Gibberella fujikuroi* by UV Treatment

Name	Phenotype	GA_3 (mg/L)	GA_3 (% of control)
G.f. aux 48	Glutamine auxotroph	820	114
G.f. aux 65	Threonine auxotroph	900	125
G.f. aux 64	Tryptophan auxotroph	690	96
G.f. nysR 277	Nystatin resistant	650	90
G.f. nysR 278	Nystatin resistant	670	93
G.f. nysR 220	Nystatin resistant	630	88
G.f. nysR 143	Nystatin resistant	660	92
G.f. cerR 428	Cerulenin resistant	750	104
G.f. cerR 439	Cerulenin resistant	780	108
G.f. cerR 454	Cerulenin resistant	680	94
G.f. cerR 461	Cerulenin resistant	695	97
G.f. nitM 371	Chlorate resistant, nit M	470	65
G.f. nitM 273	Chlorate resistant, nit M	700	97
G.f. nitM 188	Chlorate resistant, nit M	770	107
G.f. nit1 176	Chlorate resistant, nit 1	780	108
G.f. nit1 187	Chlorate resistant, nit 1	770	107
G.f. nit1 191	Chlorate resistant, nit 1	720	100
G.f. nit1 269	Chlorate resistant, nit 1	800	111
G.f. nit1 137	Chlorate resistant, nit 1	520	72
G.f. A 29	Albino mutant	600	83
G.f. A 115	Albino mutant	720	100
G.f. A 769	Albino mutant	740	103
G.f. A 770	Albino mutant	540	75
G.f. A 789	Albino mutant	540	75
G.f. 3422 (parent mutant strain)		720	100
G.f. m 567 (wild strain)		560	

genes for the molybdenum containing cofactor for nitrate reductase (*nit* M) have been isolated most often. We could find only one mutant with a block in the gene encoding nitrite reductase. Gibberellic acid production was determined for all selected mutants. For fusion experiments, only the most active strains of two phenotypes, *nit* 1 and *nit* N, were used (Table 1).

A well known strategy for selection of overproducers of secondary metabolites is the isolation of mutants, resistant to a variety of mitotic inhibitors, heavy metals, amino acid analogs, and inhibitors of specific enzymes of the interesting pathway (*18,19*). Since gibberellins are diterpenoids, we used two drugs inhibiting different steps of the terpenoid

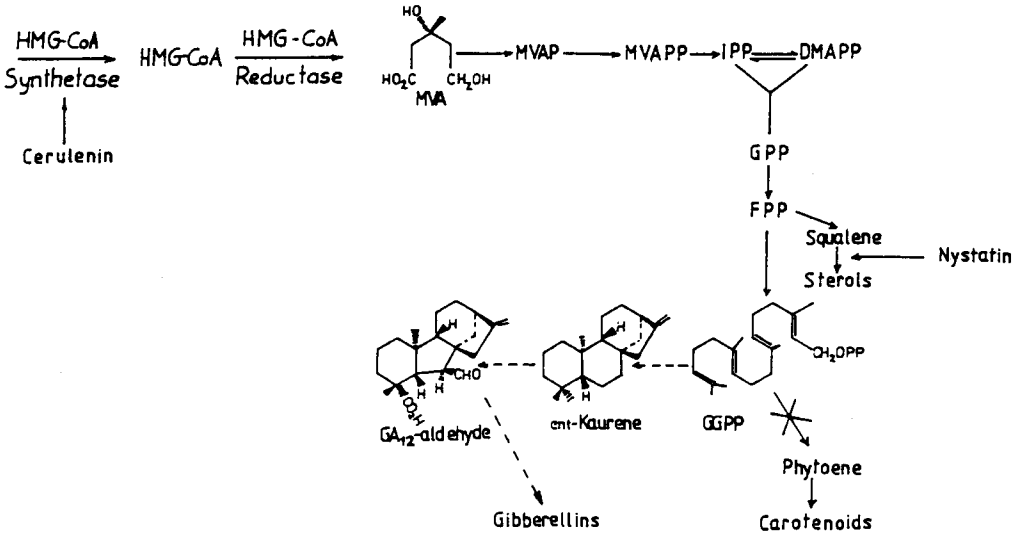

Fig. 1. Side of action of nystatin and cerulenin on the biosynthesis of gibberellins.

pathway: nystatin and cerulenin (Fig. 1). Nystatin has been shown to complex with ergosterol in the cell membrane. Nystatin-resistant mutants might be changed in the spectrum and quantity of produced sterols (*20,21*). The antibiotic cerulenin has a specific and strong inhibiting activity against the synthesis of fatty acids and sterols (*22*) and is also described as an inhibitor of HMG-CoA-synthetase, one of the key enzymes of terpenoid pathway (*3*). As shown in Table 1, we could not isolate drug-resistant mutants with a significant overproduction of gibberellic acid.

Another possible way to select overproducing mutant strains is the genetic block of carotenoid biosynthesis as a competitor to gibberellin biosynthetic pathway. The wild-type strain of *G. fujikuroi* was white on solid media but produces pigments in submerged cultivation. For better separation of wild-type colonies and pigmentless albino strains, we used a two-step mutagenesis. In the first step, we yielded stable orange mutants with a frequency of 0.02–0.04%. These mutants have growth and sporulation pattern similar to the white parent strains. The orange carotenoid pigment seems to be neurosporaxanthin as described by Avalos et al. (*24–26*). The color developed on all media independent of light. After a second UV treatment, white albino mutants were recovered (0.07–0.08%). After purification by single conidium isolation, they are stable. From all groups of mutants, the best gibberellin-producing strains were recovered for fusion experiments.

Protoplast Fusions

Protoplast fusion experiments were performed between complementary auxotrophic, *nit* 1 and *nit* M mutants, one auxotrophic and one nitrate

Table 2
Results of Protoplast Fusion Experiments
Between Different Marked Strains of *Gibberella fujikuroi*

Protoplast fusion pair	Fusion frequency (%)	Phenotype
aux 48 x aux 65	4.20	prototrophy
aux 48 x aux 64	1.90	prototrophy
aux 64 x aux 65	2.40	prototrophy
nit 1-191 x aux 48	0.07	nitrate assimilation, prototrophy
nit 1-191 x aux 65	0.30	nitrate assimilation, prototrophy
nit 1-176 x aux 65	0.40	nitrate assimilation, prototrophy
nit 1-187 x aux 48	7.50	nitrate assimilation, prototrophy
nit 1-188 x aux 48	0.20	nitrate assimilation, prototrophy
nit M-371 x nit 1-137	0.1	nitrate assimilation,
nit M-371 x nit 1-269	3.5	nitrate assimilation,
nit M-188 x nit 1-191	2.1	nitrate assimilation,
nit M-188 x nit 1-187	1.6	nitrate assimilation,
nit M-273 x nit 1-176	4.2	nitrate assimilation,
nit M-188 x cer R-439	7.50	nitrate assimilation, cerulenin resistant
nit 1-137 x cer R-428	2.60	nitrate assimilation, cerulenin resistant
nit 1-187 x cer R-428	3.20	nitrate assimilation, cerulenin resistant
nit 1-176 x cer R-454	0.20	nitrate assimilation, cerulenin resistant
cer R-454 x nys R-278	0.03	cer-, nys-double resistance
cer R-454 x nys R-143	0.10	cer-, nys-double resistance
cer R-461 x nys R-277	0.08	cer-, nys-double resistance
cer R-461 x nys R-220	0.10	cer-, nys-double resistance
A 29 x A 769	3.10	orange pigment
A 29 x A 789	2.80	orange pigment

assimilatory pathway mutant, different resistant mutants, and between albino mutants. A summary of the successful fusions is given in Table 2. Protoplast regeneration frequencies of the mutant strains varied between 35 and 70% before PEG treatment. After a 72-h incubation at 28°C, heterokaryotic colonies appeared on the agar selection plates. These could be attributable only to the regeneration of fused protoplasts. No protoplasts from the individual parental strains regenerated on selection agar.

The colonies show the typical irregular growth pattern of heterokaryons. When the colonies were transferred to complete agar, they often produced sectors of both parental classes of auxotrophy, pigmentation, nitrate assimilatory mutation, or drug sensitivity. After transfer to unstabilized minimal or selection agar, most of the randomly selected heterokaryons continued to grow slowly. However, some of the fusant colonies

Table 3
Genetic Characterization of Some Fast-Growing Fusant Colonies

Protoplast fusion pair	Number of prototrophic spores (%)	Number of sectors on MBC agar	Number of prototrophic sectors	Ploidy of fusion product
nit M-371 x nit 1-269/2	100.0	3	1	2n
nit M-371x nit 1-269/5	100.0	8	7	2n
nit M-371x nit 1-137/2	100.0	4	2	2n
nit M-188x nit 1-191/9	62.0	3	1	2n
nit M-273x nit 1-176	66.0	5	4	2n
nit M-188x nit 1-187	0.1	0	—	1n
aux 48 x aux 48 /9	100.0	2	1	2n
aux 64 x aux 48 /43	100.0	5	1	2n
aux 65 x aux 48 /44	2.0	0	—	1n
nit M-188x aux 48 /5	65.0	4	3	2n
nit 1-176 x aux 65 /8	50.0	3	1	2n
nit 1-176 x aux 65 /14	0.1	0	—	1n

developed faster and with wild-type morphology on selection agar. The amount of fast-growing fusant colonies could be increased by multistep cultivation on minimal agar supplemented with camphor as described by Kirimura et al. (*1*) Protoplast fusion between auxotrophic and nitrate assimilatory pathway mutants proved to be the most successful.

Protoplast fusion between drug-resistant mutants resulted in only a few heterokaryons. The colonies were slow-growing even after subculturing and had an irregular shape. Most of the fusion products lost the ability to grow on selection medium after some transfers. No sectors of more vigorous growth appeared after 3 wk of culture on MM supplemented with nystatin and cerulenin.

For genetic characterization of the fast-growing prototrophic fusant strains, the microconidia were plated on Czapek-Dox minimal agar and CM to check the frequency of prototrophic spores. To determine whether the prototropic microconidia are diploid or haploid recombinants, MBC was used to induce haploidization. After 2 wk on MBC agar, the genetic stability of some of the prototrophic colonies was lost. A total of 28 fusant colonies, mostly isolated from MM supplemented with camphor, segregated sectors. The segregants had various phenotypes in morphology and nutritional markers. Many segregants were prototrophic, but single auxotrophs and one double auxotroph also could be isolated.

These strains segregating sectors are diploid (aneuploid) fusants. The results of these experiments are given in Table 3. Generally, the number of stable diploids resulting from fusions between different auxotrophic or nitrate assimilatory pathway mutants is about 5% of all fusant colonies

examined. From 10 possible combinations between five albino strains, only two pairings (A 29·A 769 and A 29·A789) gave orange colonies on CM and MM. In these cases, the mutants carry blocks in different steps of the carotenoid biosynthetic pathway. On the other hand, eight fusion pairs did not develop orange-pigmented colonies. Therefore, they seem to be mutated in the same gene of carotenoid biosynthesis.

Gibberellic Acid Formation

Gibberellic acid productivities of all fast-growing stable fusants and prototrophic segregants were determined and compared with those of the parent strains. As shown in Table 4, the best results were obtained for hybrid strains. On the other hand, no haploid segregants had a higher productivity than the diploid or parent strains.

DISCUSSION

There are many studies concerning intraspecific protoplast fusion of fungal strains used in industry (*1,2,12,27*). However, no one has reported protoplast fusion between gibberellin-producing mutants of *G. fujikuroi*. The experiments here show that in addition to auxotrophic mutants, a wide range of genetically marked strains can be used for protoplast fusion.

Mutants blocked in the nitrate assimilation system, conferring chlorate resistance, are good producers of gibberellic acid in many cases. They can be crossed with other chlorate-resistant strains blocked in a different step of the nitrate assimilatory pathway or with auxotrophic strains. In general, the highest fusion frequencies were obtained for hybridization between complementary auxotrophic, complementary nitrate assimilation mutants, and one auxotrophic and one nitrate assimilatory pathway mutant.

Furthermore, complementation between gibberellin-overproducing albino mutants did occur, and the formation of orange-pigmented fusant colonies was a visible indicator of heterokaryosis. Puhalla (*28*) described heterokaryosis in albino strains of *Fusarium oxysporum* following hyphal anastomosis. However, in our experiments, only two pairings between albino strains restored their ability to produce carotenoids. They should be blocked at different steps of carotenoid biosynthesis. Cerda-Olmedo (*29*) analyzed some albino strains of *G. fujikuroi* and found them to be blocked at the gene for prephytoene-pyrophosphate-synthetase. A second possible block could be in the gene coding for phytoene-synthetase (Fig. 1). Most of the orange fusants produced less gibberellic acid than the parental albino strains, probably caused by recovery of the carotenoid biosynthesis.

Protoplast fusion between nystatin- and cerulenin-resistant mutants occurred rarely. The fusant colonies often showed atypical morphology,

Table 4
Gibberellic Acid Productivities of Some Fusant and Sector Strains in Standardised Shaking Culture

Protoplast fusion pairs		Ploidy	GA_3 (mg/l)	GA_3 (% of the best parental strain)
nit M-371 x nit 1-269	/1	2n	480	60
	/2	2n	780	98
	/3	2n	460	58
	/4	2n	720	90
	/5	2n	950	119
nit M-371 x nit 1-269/5	/A	1n	610	76
	/C	1n	700	88
	/D	1n	510	64
	/F	1n	520	65
	/G	1n	710	89
nit M-371 x nit 1-137	/1	2n	730	140
	/2	2n	910	175
nit M-188 x aux 48	/5	2n	440	54
	/28	2n	600	73
	/30	2n	620	76
	/32	2n	850	104
nit M-188 x aux 48	/A	1n	410	50
	/B	1n	390	48
	/C	1n	680	83
nit 1-187 x aux 48	/1	2n	680	83
	/2	2n	780	95
	/3	2n	920	112
aux 48 x aux 65	/17	2n	820	100
	/22	2n	790	98
	/31	2n	480	59
	/44	2n	720	88
	/49	2n	900	110
A 29 x A 769	/1	2n	870	106
	/2	2n	690	84
	/3	2n	620	76
A 29 x A 789	/1	2n	590	72
	/2	2n	720	88
	/3	2n	610	74
Control parental strains:				
nit M-371			470	
nit M-188			770	
nit 1-269			800	
nit 1-187			770	
nit 1-137			520	
aux 48			820	
aux 65			800	
A 29			760	
A 769			840	
A 789			820	

grew slowly, or lost their double-resistant phenotype. The instability of drug-resistance in fusant colonies may be caused by the semidominant or recessive character of these mutations as described for *Dictyostelium discoideum* (*30*). After spontaneous haploidization occurring on CM, subcultures showed one or the other of the parental resistance phenotypes.

In general, protoplast fusion results in heterokaryons, stable diploids, or haploid recombinants. A camphor treatment of fusant colonies increased the frequency of diploids significantly, as described for *A. niger,* too (*31*). The number of stable diploids was about 5% of all fast-growing fusant colonies. They formed auxotrophic and prototrophic sectors on agar supplemented with the haploidizing agent MBC.

If strains are crossed, we have no knowledge about the influence of fusion process on the gibberellin biosynthetic pathway, and it is a matter of chance whether the recombinants are good producers. As shown in this paper, some of the diploid strains had higher productivities than each parental strain and the haploid segregants. However, most of the diploid and haploid recombinant strains exhibited lower gibberellin titers than the parent strains. It was found that certain crosses (e.g., *nit* M-371·*nit* 1-269) are more profitable than others. Therefore, success in strain improvement by protoplast fusion depends on finding useful crosses and concentrating upon them. The same situation has been observed in mutation experiments for strain improvement.

REFERENCES

1. Kirimura, K., Yaguchi, T., and Usami, S. (1986), Intraspecific protoplast fusion of citric acid-producing strains of Aspergillus niger. *J. Ferment. Technol.* **64,** 473–479.
2. Calam, C. T., Daglish, L. B., and McCann, E. P. (1976), Penicillin: tactics in strain improvement. *Genetics of Industrial Microorganisms,* McDonald, ed., pp. 273–287.
3. Kurth, R. and Demain, A. L. (1984), The impact of the new genetics on antibiotic production. *Biotechnology of Industrial Antibiotics,* Vandamme, E. J., ed., Marcel Dekker, New York, pp. 781–789.
4. Jefferys, E. G. (1970), The gibberellin fermentation. *Adv. Appl. Microbiol.* **13,** 283–316.
5. Brückner, B., Blechschmidt, D., and Schubert, B. (1989), Fusarium moniliforme SHELD—a fungus producing a broad spectrum of bioactive metabolites. *Zbl. Bakteriol.* **144,** 3–12.
6. Imshenetsky, A. A. and Ulyanova, O. M. (1962) Experimental variability in Fusarium moniliforme Sheld leading to the formation of gibberellins. *Nature* **195,** 62,63.
7. Erokhina, L. I. and Efremov, B. D. (1970), Biochemical mutants of Fusarium moniliforme (Sheld). *Genetika* **6,** 170–172.
8. Vass, R. C. and Jefferys, E. G. (1979), Gibberellic acid, *Economic Microbiology: Secondary Products of Metabolism,* vol. 3, Rose, A. H., ed., Academic, London, pp. 421–433.

9. Calam, C. T., Daglish, L. B., and Gaitskell, W. S. (1973), Hybridisation experiments with Penicillium patulum and Fusarium moniliforme, *Genetics of Industrial Microorganisms*, Vanek, Z., Hostalek, Z., and Cudlin, J., eds., Academia Prague, pp. 265–282.
10. Brückner, B., May, R., and Loeffler, B., (1990), Formation and regeneration of protoplasts from Gibberella fujikuroi. *J. Basic Microbiol.* **30,** 147–152.
11. Dales, R. B. G. and Croft, J. H. (1977), Protoplast fusion and the isolation of heterokaryons and diploids from vegetatively incompatible strains of Aspergillus nidulans. *FEMS Microbiol. Lett.* **1,** 201–204.
12. Reymond, P. and Fevre, M. (1986) Recombination following protoplast fusion of penicillium strains used in the dairy industry. *Enzyme Microb. Technol.* **8,** 41–44.
13. Darken, M. A., Jensen, A. L., and Shu, P. (1959), Production of gibberellic acid by fermentation. *Appl. Microbiol.* **7,** 301–303.
14. Leslie, J. (1983), Some genetic techniques for Gibberella zeae. *Phytopathology* **73,** 1005–1008.
15. Correll, J. C., Klittich, C. J. R., and Leslie, J. F. (1987), Nitrate nonutilizing mutants of Fusarium oxysporum and their use in vegetative compatibility tests. *Phytopathology* **77,** 1640–1646.
16. Timberlake, W. T. and Marshall, M. A. (1989), Genetic regulation of development in Aspergillus nidulans. *TIG* **4,** 162–169.
17. Kikuchi, M. (1980), Application of genetics for strain improvement in industrial microorganisms. *Biotechnol. Bioeng.* **22,** 195–208.
18. Elander, R. P. (1980), New genetic approaches to industrial important fungi. *Biotechnol. Bioeng.* **22,** 49–61.
19. Rowlands, R. T. (1984), Industrial strain improvement: rational screens and genetic recombination techniques. *Enzyme Microb. Technol.* **6,** 290–300.
20. Molzahn, S. W. and Woods, R. A. (1972), Polyene resistance and the isolation of sterol mutants in Saccharomyces cerevisiae. *J. Gen. Microbiol.* **72,** 339–348.
21. Luengo, J. M., Revilla, G., Lopez, M. J., Villanueva, J. R., and Martin, J. F. (1979), Penicillin production by mutants of Penicillium chrysogenum resistant to polyene macrolide antibiotics. *Biotechnol. Lett.* **1,** 233–238.
22. Iwai, Y., Awaya, J., Kesado, T., Yamada, H., Omura, S., and Hata, T. (1973), Selective production of cerulenin by Cephalosporium caerulens KF-140. *J. Ferment. Technol.* **51,** 575–581.
23. Norman, S. H. and Poling, S. M. (1988), Abscisic acid biosynthesis in Cercospora rosicola: Sensibility to inhibitors of sterol biosynthesis. *Agric. Biol. Chem.* **5,** 1309, 1310.
24. Avalos, J., Casadesus, J., and Cerda-Olmedo, E. (1985), Gibberella fujikuroi mutants obtained with UV-radiation and N-methyl-N'-nitro-N-nitrosoguanidine. *Appl. ENviron. Microbiol.* **49,** 187–191.
25. Avalos, J. and Cerda-Olmeda, E. (1986), Chemical modification of carotenogenesis in Gibberella fujikuroi. *Phytochem.* **25,** 1837–1841.
26. Avalos, J. and Cerda-Olmeda, E. (1987), Carotenoid mutants of Gibberella fujikuroi. *Curr. Genet.* **11,** 505–511.
27. Hamlyn, P. F. and Ball, C. (1979), Recombination studies with Cephalosporium acremonium. *Genetics of Industrial Microorganisms*, Sebek, O. K. and Laskin, A. I., eds., *Amer. Soc. Microbiol.*, Washington, DC, pp. 185–191.

28. Puhalla, J. E. (1985), Classification of strains of Fusarium oxysporum on the basis of vegetative compatibility. *Can. J. Bot.*, **63,** 179–183.
29. Cerda-Olmedo, E. (1988), Production of carotenoids with fungi, *Biotechnology of Vitamins, Pigments and Growth Factors,* Vandamme, E. J., ed., Elsevier Applied Science, pp. 27–42.
30. Kasbekar, D.P., Madigan, S., and Katz, E. R. (1983), Use of nystatin-resistant mutations in parasexual genetic analysis in dictyostelium discoideum. *Genetics* **104,** 271–277.
31. Kirimura, K., Nakajima, I., Lee, S. P., Kawabe, S., and Usami, S. (1988), Citric acid production by the diploid strains of Aspergillus niger obtained by protoplast fusion. *Appl. Microbiol. Biotechnol.* **27,** 504–506.
32. Klittich, C. J. R. and Leslie, J. (1988), Nitrate reduction mutants of Fusarium moniliforme (Gibberella fujikuroi). *Genetics,* **118,** 417–423.
33. Kölblin, R., Brückner, B., Blechschmidt, D., and Fischer, W. (1991), Mutagenesis of Gibberella fujikuroi. *J. Basic Microbiol.* (in press).
34. Correll, J. C., Klittich, C. J. R., and Leslie, J. F. (1989), Heterokaryon self-incompatibility in Gibberella fujikuroi (Fusarium moniliforme). *Mycol. Res.* **93,** 21–27.