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Strain-Specific Synthesis of Mycophenolic Acid by *Penicillium roqueforti* in Blue-Veined Cheese

GÜNTER ENGEL,[1] KARL ERNST VON MILCZEWSKI,[1] DIETER PROKOPEK,[2] AND MICHAEL TEUBER[1]*

Institute of Microbiology[1] and Institute of Process Engineering,[2] Federal Dairy Research Center, D-2300 Kiel 1, West Germany

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Twenty of 80 strains of *Penicillium roqueforti* were able to produce up to 600 mg of mycophenolic acid (MPA) liter^{-1} in 2% yeast extract–5% sucrose broth. Sixty-two of these strains had been isolated from the main blue-veined cheese varieties of western Europe or from starter cultures. Of these 62 dairy strains, only 7 had MPA-producing potential in vitro. These seven strains had all been isolated during the period 1975 to 1981 from the blue cheese of one individual factory. In cheese from the market, MPA (up to 5 mg kg^{-1}) was only found in samples of this same factory. With MPA-producing and -nonproducing strains for the experimental manufacture of blue cheese, MPA synthesis in cheese was only detected with strains which form MPA in yeast extract-sucrose broth. The maximum MPA level at 4 mg kg^{-1} was similar to that in commercial cheese. Toxicity of MPA was tested with two established human cell lines (Detroit 98 and Girardi Heart) and one established pig kidney cell line (AmII).

Mycophenolic acid (MPA) (Fig. 1) is a toxic metabolite produced by several *Penicillium* species such as *P. stoloniferum* (2), *P. viridicatum* (4), *P. brevicompactum* (6), and some strains of *P. roqueforti* (11). In mammals, MPA is rapidly absorbed from the digestive tract, and after conjugation it is excreted as MPA-glucuronide in the urine (15). The acute and chronic toxicity has been described for rats (5). In some blue-veined cheeses, MPA has been reported in concentrations up to 15 mg kg^{-1} (12).

We have developed a thin-layer chromatographic method to separate MPA from any contaminating compounds in cheese extracts with a limit of detection of 75 μg kg^{-1}. With this method, 32 samples of the major European blue cheeses were examined. In addition, 80 strains of *P. roqueforti* isolated from starter cultures, cheese, and other foods were investigated for their ability to produce MPA. With MPA-producer and -nonproducer strains, experimental blue-veined cheese was made and analyzed for MPA content. With cell culture lines of human origin, the limits of sublethal toxicity were evaluated.

MATERIALS AND METHODS

Origin and maintenance of *P. roqueforti* strains. The strains investigated constitute the *P. roqueforti* culture collection of the Institute of Microbiology of the Federal Dairy Research Center in Kiel, West Germany. The sources and data of acquisition are listed in Table 1. The microscopic and macroscopic properties of all strains agree with the classical identification as previously reported (9). In addition, all strains grow on the selective acetic acid–Czapek-Dox medium (9).

Stock cultures were prepared on 2% malt extract agar (E. Merck AG, Darmstadt, West Germany; no. 5398) at pH 5.6. After incubation at 25°C for 7 days, the cultures were stored at 4 to 8°C. Conidia from 7-day-old subcultures grown at 25°C served as inocula for MPA production.

Production of reference MPA. MPA-producing *P. stoloniferum* D310 was obtained from P. Lafont (Le Vesinet, France). From 25 liters of 2% yeast extract–5% sucrose broth (7), 10 g of crystalline MPA (mp, 141°C) was isolated. The infrared spectrum of this material, recorded in KBr pellets, showed the characteristic absorption peaks of MPA (12).

Production of MPA by *P. roqueforti* in 2% yeast extract–5% sucrose broth. For estimation of the MPA-producing potential of *P. roqueforti*, 0.1 ml of conidial suspension (1 × 10^8 to 3 × 10^8 conidia ml^{-1}) of each strain was added to 100 ml of liquid medium in 500-ml Erlenmeyer flasks. Incubation was for 10 days at 20°C in the dark.

Production of MPA in blue-veined cheese. For each of the *P. roqueforti* strains to be tested, two loaves of

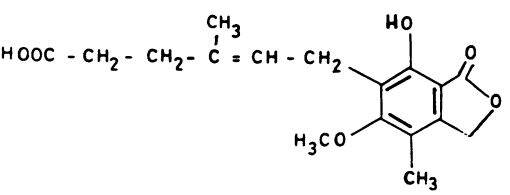

FIG. 1. Structural formula of MPA.

cheese were produced by a standard method described elsewhere (8). The ripened cheeses had a mean NaCl content of 3.7%, corresponding to 7.0% in the aqueous phase. The MPA content was determined after 30, 50, 65, and 80 days from two samples taken from the cheese interior and one sample from the cheese rind.

Extraction of MPA from nutrient medium. The medium was acidified with HCl to pH 1.5, mixed with 100 ml of acetonitrile, and homogenized in an Ultraturrax to macerate the mycelium. After addition of 50 ml of chloroform and further homogenization, the mixture was filtered through paper and transferred into a funnel. The organic phase was removed, the aqueous phase was once more extracted with 50 ml of $CHCl_3$, and the combined organic phases were dried over sodium sulfate. The solvents were removed in vacuo in a rotary evaporator. The residue was taken up in 1 ml of $CHCl_3$ plus 1 ml of acetonitrile.

Extraction of MPA from cheese (13). A 25-g portion of cheese was homogenized with 50 ml of 5% aqueous NaCl in an ultraturrax for 2 min. The pH was adjusted to 6 with 1 N acetic acid. After addition of 150 ml of methanol-$CHCl_3$ (1:1, vol/vol), the mixture was homogenized for another 3 min and filtered, and the filtrate was stored overnight at −20°C. The precipitated casein was removed by centrifugation (10 min, 5,000 × *g*). The supernatant was evaporated to about 100 ml in a rotary evaporator, quantitatively transferred into a funnel, and extracted three times with 100 ml of *n*-hexane each time. This defatted aqueous phase was consecutively extracted with two 100-ml portions of $CHCl_3$, 100 ml of $CHCl_3$-ethylacetate (1:1, vol/vol), and finally 100 ml of ethylacetate. The combined extracts were dried with sodium sulfate, evaporated to dryness, dissolved in $CHCl_3$, transferred quantitatively into a screw-cap vial, evaporated under nitrogen to dryness, and dissolved in 125 μl of $CHCl_3$.

Quantitative determination of MPA by thin-layer chromatography. (i) Nutrient medium. A 10-μl amount of extract or a properly diluted medium was spotted onto silica gel (0.25-mm) plates (G1500 F_{254} with fluorescent indicator; Schleicher & Schüll, Dassel, Germany), 20 by 20 cm. The chromatograms were developed with the solvent system ethylether–*n*-hexane–formic acid (60:20:0.4, vol/vol/vol) until a distance origin-solvent front of 15 cm was reached (14).

(ii) Cheese extracts. A 10-μl amount was spotted onto silica gel plates and separated by two-dimensional chromatography (16). As solvent systems for the first dimension, *n*-hexane–ethylacetate (94:6, vol/vol) followed by ethylether–*n*-hexane–formic acid (60:20:4, vol/vol/vol) were applied; for the second dimension, $CHCl_3$-acetone-water (93:7:1, vol/vol/vol) was used (13).

MPA was then directly quantitated on the developed thin-layer plates, using a double-beam spectrodensitometer (Schoeffel, Westwood, N.J.), by UV remission at 250 and 300 nm, respectively (7). The identity of MPA was checked by scanning the UV spectrum of the suspected spot with the same instrument by fluorescence quenching of the fluorescence indicator of the plates and by fluorescence after spraying of the plates with ammonia (7, 11).

Cell culture assay for toxicity. The cell lines Detroit 98 (CCL 18, human origin) and Girardi Heart (CCL 27, human origin) were grown in Hanks medium 199 from subcultures 118 to 167 and 520 to 572, respectively. In addition, an established pig kidney cell line, AmII (subculture 71), was used as previously described (13). MPA was dissolved in dimethyl sulfoxide and diluted 1:100 with cell culture medium before addition to the cells during seeding. MPA was present during the entire incubation period.

RESULTS

Detection of MPA. The extraction and chromatography procedures used have limits of detection for MPA of 200 μg $liter^{-1}$ in the case of yeast extract-sucrose broth and 75 μg kg^{-1} in the case of cheese. Isolated MPA added to liquid medium and MPA-free cheese can be recovered at 75 to 100% after 24 h of storage at room temperature in the dark at levels of 250 and 75 μg kg^{-1}. Figure 2 demonstrates that the two-dimensional thin-layer chromatography system used allows a clear separation of MPA from contaminating UV-absorbing substances in cheese extracts. This holds true for blue-veined cheese containing MPA from the used starter culture and for cheese contaminated with isolated, crystalline MPA in vitro.

Incidence of MPA in West European blue-veined cheese varieties. To confirm the work of Lafont's (11, 14) laboratory on the incidence and level of MPA in West European blue-veined cheese, we analyzed the major corresponding cheese varieties available on the German market. For this purpose, we used the above-described quantitative two-dimensional thin-layer chromatography, which allows an unequivocal separation and identification of MPA. Table 2 demonstrates that MPA was detected only in four specimens (no. 8 to 11) of roquefort cheese, which is produced from sheep's milk. These cheeses all originated from the same factory. Another roquefort cheese (no. 12) stemming from another cheese factory was free of MPA.

Potential of *P. roqueforti* strains to synthesize MPA. To prove that the starter culture is responsible for any MPA detectable in blue-veined cheese, we investigated the MPA-synthesizing potential of all 80 *P. roqueforti* strains at our disposal. Seventeen strains had been obtained from 1975 and onwards from colleagues and culture collections. The others have been isolated and identified in our own laboratory from cheeses and starter cultures. MPA production was determined in 2% yeast extract–5% sucrose broth, which gives high yields of secondary metabolites in *P. roqueforti* (9). Twenty of 80 strains exhibited a significant level of MPA in broth. These strains originated mainly from other culture collections. In our own laboratory, MPA-producing strains could only be isolated from genuine roquefort cheeses of one individual cheese factory (or distributor). Strains of this type have been found in 1975 as well as in 1981

TABLE 1. Production potential for MPA of the *P. roqueforti* strains deposited in the collection of the Institut of Microbiology of the Federal Dairy Research Center, Kiel[a]

No. assigned in Kiel collection	Geographic origin/source	Substrate of original isolation	Yr of acquisition	MPA (mg liter^{-1})
6753	CBS Baarn (Holland), no. 28067		1972	2.0
6766	J. Harwig (Canada), ATCC 34905	Danablu	1977	24
6767	S. Moreau (France), 8 US 76		1977	28
6768	H. K. Frank (Germany), NRRL 849	Roquefort	1976	18
6886	L. Leistner (Germany), Sp 390	Raw sausage	1978	4
6887	L. Leistner (Germany), Sp 411		1978	30
6888	L. Leistner (Germany), Sp 455	Raw sausage	1978	600
6889	L. Leistner (Germany), Sp 581	Cured meat	1978	360
6890	L. Leistner (Germany), Sp 626	Raw sausage	1978	0
6891	H. K. Frank (Germany), Sp 735		1978	0
6892	L. Leistner (Germany), Sp 739	Oat creme	1978	0
6893	L. Leistner (Germany), Sp 740, ATCC 44165	Oat creme	1978	1.4
6894	H. K. Frank (Germany), Sp 765, CMJ 34909		1978	2.4
6895	L. Leistner (Germany), Sp 858	Gouda cheese	1978	0
6896	L. Leistner (Germany), Sp 859	Gouda cheese	1978	0
6897	L. Leistner (Germany), Sp 860	Gouda cheese	1978	16
6899	L. B. Bullerman (United States), PDA 6-13	Cheddar cheese	1978	140
6813	Denmark	Blue Castello	1975	0
6845	Denmark	Danablu	1970	0
6859	Denmark	Danablu	1977	0
6860	Denmark	Blue Castello	1976	0
6862	Denmark	Blue cheese	1976	0
6869	Denmark	Blue cheese	1976	0
6898	Denmark	Blue cheese	1978	0
68106	Denmark	Danablu	1978	0
68111	Denmark	Blue Castello	1978	0
68130	Denmark	Blue Castello	1980	0
68139	Denmark	Blauer Kommandör	1981	0
6815	France	Roquefort	1975	64
6816	France	Bresse Bleu	1975	0
6847	France	Bresse Bleu	1970	0
6858	France	Roquefort	1976	20
6863	France	Pipi Bleu	1976	0
6864	France	Roquefort	1976	14
6865	France	Roquefort	1976	40
68104	France	Roquefort	1978	40
68107	France	Roquefort	1978	40

(see Table 1). It is clear that some of the strains may be identical since cheeses and starter cultures from individual producers have been sampled on several occasions during a 6-year period. Obviously, there exists a family of MPA-producing and a family of nonproducing strains. This marker seems to be stable under the industrial production conditions during the covered time period.

Production of MPA in experimental blue-veined cheese. To obtain final proof that MPA in blue-veined cheese is synthesized by the *P. roqueforti* strain used as a starter culture, we prepared blue cheese with producer and nonproducer strains previously isolated from cheese and starter cultures. Table 3 provides the experimental evidence that only strains able to produce MPA in the yeast extract-sucrose broth also produce this compound in cheese under the conditions of industrial cheese fermentation. However, the level of MPA in cheese is about 50 to 100 times lower than that in broth. These data also allow the conclusion that the "nonproducer" strains must produce less than 1/1,000 of the level of producer strains if there should be a residual production below the limit of detection

TABLE 1—*Continued*

No. assigned in Kiel collection	Geographic origin/source	Substrate of original isolation	Yr of acquisition	MPA (mg liter^{-1})
68108	France	Bresse Bleu	1978	0
68128	France	Roquefort	1980	24
68136	France	Bresse Bleu	1981	0
68140	France	Bresse Bleu	1981	0
68141	France	Le bon Livreur	1981	0
6814	Germany	Edelpilz cheese	1975	0
6846	Germany	Edelpilz cheese	1970	0
6861	Germany	Edelpilz cheese	1976	0
6866	Germany	Bavaria Blue	1976	0
6867	Germany	Edelpilz	1976	0
68101	Germany	Bavaria Blue	1978	0
68102	Germany	Edelpilz	1978	0
68110	Germany	Edelpilz	1978	0
68138	Germany	Blue Alb	1981	0
68133	Great Britain	Blue stilton A	1980	0
68134	Great Britain	Blue stilton B	1981	0
68135	Great Britain	Blue stilton C	1981	0
6817	Italy	Gorgonzola A	1975	0
6818	Italy	Gorgonzola B	1975	0
6870	Italy	Gorgonzola C	1976	0
68105	Italy	Gorgonzola D	1978	0
68109	Italy	Gorgonzola D	1978	0
68131	Italy	Gorgonzola B	1980	0
68132	Italy	Gorgonzola E	1980	0
68137	Denmark	Starter culture	1981	0
6829	Germany	Starter culture A	1975	0
6830	Germany	Starter culture B	1975	0
6848	Germany	Starter culture C	1973	0
6850	Germany	Starter culture B	1973	0
6873	Germany	Starter culture B	1977	0
6874	Germany	Starter culture B	1977	0
6875	Germany	Starter culture B	1977	0
6876	Germany	Starter culture B	1977	0
6877	Germany	Starter culture D	1977	0
6878	Germany	Starter culture E	1977	0
6879	Germany	Starter culture F	1977	0
6880	Germany	Starter culture G	1977	0
68129	Germany	Starter culture A	1981	0
6800	Italy	Starter culture A	1975	0
6821	Italy	Starter culture B	1975	0

[a] MPA was determined after 10 days of growth at 20°C in 2% yeast extract–5% sucrose broth by quantitative one-dimensional thin-layer chromatography. The limit of detection was 0.2 mg liter of medium^{-1}.

(0.2 mg liter of broth^{-1}). For that reason, we consider this "zero production."

Toxicity of MPA towards human cell lines. Human cell cultures have been used in our laboratory to provide a rapid screening method for the eventual mycotoxin content of food additives, especially enzymes produced by fungi (13). In this test, cytocidal, growth inhibitory, and attachment inhibitory effects of mycotoxins may be detected. In this system, 100 μg of MPA ml^{-1} had no effect on the attachment and stretching of the investigated Girardi Heart and AmII cell lines. There was a partial reduction in the attachment rate of cells of the Detroit 98 line at this MPA concentration. After 6 days of incubation of the cell cultures in the presence of MPA, cytocidal effects were observed at >100, 33, and 4 to 11 μg ml^{-1} with AmII, Detroit 98,

TABLE 2. MPA content of blue-veined cheeses produced in Western Europe[a]

Country of origin (in alphabetical order)	Date of purchase	Kind of cheese	MPA content (μg/kg)	*P. roqueforti* strain (no.) isolated from this cheese[b]
Denmark	Aug. 1980	Danablue	<75	
	Sept. 1980	Danablue	<75	
	Sept. 1980	Blue Castello	<75	68130
	Jan. 1981	Blue Castello	<75	
	Feb. 1981	Danablue	<75	
	Feb. 1981	Danablue	<75	
	Feb. 1981	Blauer Kommandör	<75	68139
France	Aug. 1980	Roquefort	250	
	Aug. 1980	Roquefort	5,000	68128
	Jan. 1981	Roquefort	5,000	
	Feb. 1981	Roquefort	3,250	
	Feb. 1981	Roquefort	<75	68141
	Aug. 1980	Bresse bleu	<75	
	Sept. 1980	Bresse bleu	<75	
	Jan. 1981	Bresse bleu	<75	68136
	Feb. 1981	Bresse bleu	<75	68140
Germany	Aug. 1980	Edelpilz	<75	
	Sept. 1980	Edelpilz	<75	
	Jan. 1981	Edelpilz	<75	
	Feb. 1981	Edelpilz	<75	
	Feb. 1981	Edelpilz	<75	
	Sept. 1980	Processed	<75	
	Sept. 1980	Bavaria blue	<75	
	Jan. 1981	Bavaria blue	<75	
Great Britain	Jan. 1981	Blue stilton	<75	68133
	Jan. 1981	Blue stilton	<75	68134
	Jan. 1981	Blue stilton	<75	68135
Italy	Sept. 1980	Gorgonzola	<75	68131
	Sept. 1980	Gorgonzola	<75	68132
	Jan. 1981	Gorgonzola	<75	
	Jan. 1981	Gorgonzola	<75	
	Feb. 1981	Gorgonzola	<75	

[a] Limit of detection was 75 μg of MPA in 1 kg of cheese (see text).
[b] Number assigned to the isolated strains in the collection of the Institute of Microbiology of the Federal Dairy Research Center in Kiel (see also Tables 1 and 3).

and Girardi Heart, respectively. Growth inhibition was noted at levels of 4, 1.2 to 4, and 1.2 μg ml^{-1} for the respective cell lines.

DISCUSSION

In our experiments, there was only one special blue-veined cheese variety which contained up to 5 mg of MPA kg^{-1} which came from one individual factory or distributor. Only *P. roqueforti* strains isolated from this cheese produced MPA in an artificial medium or in experimental blue cheese. All other strains isolated from different blue-veined cheeses and commercial starter cultures did not synthesize MPA under the investigated conditions. This behavior of strains has not changed during the observation period from 1975 to 1981. The highest MPA-producing potential (600 mg $liter^{-1}$), however, has been detected in two strains originally isolated from molded raw sausages and cured meat as contaminants.

To aid a realistic evaluation of any eventual toxicological risk of the consumption of MPA-containing cheese, it is useful to recall the main experimental results pertinent to humans. A daily oral application of 40 to 80 mg kg^{-1} in the treatment of psoriasis for 12 weeks did not induce harmful effects (10). At daily doses of 150 mg kg^{-1}, rhesus monkeys, however, developed hypoplastic anemia and severe intestinal disor-

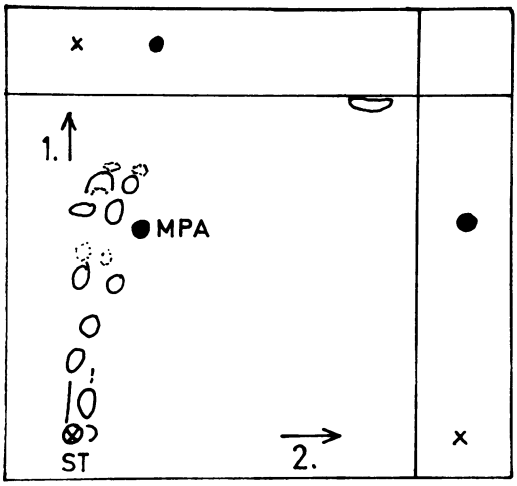

FIG. 2. Two-dimensional thin-layer chromatogram of an MPA-containing extract from a blue-veined cheese. ST, Start position; 1, first dimension; 2, second dimension; x, start positions for reference mycophenolic acid. For further details see the text.

ders. In contrast, at 50 mg kg^{-1} day^{-1}, the monkeys were without adverse effects (5). In rats, a moderate chronic toxicity was observed at a daily oral intake of 15 mg kg^{-1} (5). Large differences in the response of individual animals to MPA seem to be caused by the difference in the rate of conversion into the glucuronide form, which is easily excreted in the urine (1).

If we assume a "no effect" level for human cells at 0.5 μg ml^{-1} in vitro (~0.5 mg kg^{-1} in vivo), a 70-kg human could consume 35 mg of MPA. This means a consumption of 7 kg of blue-veined cheese containing 5 mg of MPA kg^{-1}, the highest value detected in cheese on the German market.

In view of the low incidence of MPA-containing cheeses on the market, the low content of MPA, and the relatively low consumption of this type of cheese, no immediate toxicological risk is envisaged. In addition, starter cultures are available which produce no MPA.

TABLE 3. Production of MPA by *P. roqueforti* in experimental blue-veined cheese and in 2% yeast extract–5% sucrose broth at 8 and 15°C[a]

Production in:	MPA in cheese (mg · kg^{-1}) and broth (mg · $liter^{-1}$) after incubation times of:			
	30 days	50 days	65 days	80 days
Strain 6865				
Broth at 8°C	135	245	245	234
Broth at 15°C	115	220	190	204
Cheese at 8°C				
Surface	0.5	0.9	0.8	0.2
Interior	0.6	0.3	0.2	0.3
Cheese at 15°C				
Surface	0.6	0.4	0.6	0.7
Interior	0.4	0.2	0.3	0.5
Strain 68104				
Broth at 8°C	165	215	210	280
Cheese at 8°C				
Surface	NT[b]	5.0	2.5	0.2
Interior	0.5	1.8	1.7	0.9
Cheese at 15°C				
Surface	NT	1.3	0.5	0.2
Interior	2.2	2.7	2.5	0.2
Strain 68128				
Broth at 8°C	120	250	305	260
Cheese at 8°C				
Surface	0.1	1.0	1.2	1.4
Interior	0.1	0.9	2.5	3.0
Cheese at 15°C				
Surface	1.0	0.4	0.7	0.5
Interior	4.0	1.6	0.5	0.6
Strains 6830 and 68129				
Broth at 8 and 15°C	ND[c]	ND	ND	ND
Cheese at 8 and 15°C (surface and interior)	ND[d]	ND	ND	ND

[a] For origin of strains, see Tables 1 and 2.
[b] NT, Not tested.
[c] ND, Not detected; limit of detection, 0.2 mg $liter^{-1}$.
[d] ND, Not detected; limit of detection, 0.075 mg kg^{-1}.

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