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Characterization of *Penicillium roqueforti* strains used as cheese starter cultures by RAPD typing

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Abstract

Seventy-six strains of *Penicillium roqueforti* used as starter cultures for mould ripened blue cheeses have been analysed for their RAPD genotype by using three different primers. A comparison of the RAPD patterns within each primer group revealed that the genetic constitution of the strains was similar, as most of the strains showed very similar overall patterns. Despite these similarities with each primer, distinct RAPD genotype groups could be identified. With one of the primers, it was possible to detect two heteropolymorphic DNA regions resulting in 13 different groups. With the other two primers, three or four groups could be identified. Between the groups of the different primers marked correspondence with respect to strain distribution could be observed, indicating that the polymorphisms detected by the primers were not independent. The RAPD patterns were compared to the production of secondary metabolites. A correlation was observed between the RAPD patterns of all primers and the production of mycophenolic acid. In addition, one of the primer (ari1) was able to distinguish between *P. roqueforti* strains producing larger or smaller numbers of metabolites.

Keywords: RAPD; *Penicillium roqueforti*; Blue cheese; Secondary metabolites

1. Introduction

Penicillium roqueforti is used as a starter culture for the production of fermented blue cheese, like Danablu, Gorgonzola, Roquefort and Stilton (Marth, 1987). It is well adapted to the specific food environment and due to its lipolytic and proteolytic activity it contributes importantly to the flavour formation of blue cheese (Gripon, 1993). Because of the different characters of the existing types of blue cheeses, *P. roqueforti* strains with different technological properties are needed. The technological properties of a given *P. roqueforti* strain are determined by its genetic constitution. The RAPD technique (random amplified polymorphic DNA) is a powerful tool for determining genetic differences of various microorganisms (Williams et al., 1990). Depending on the type of random primer used, the resulting RAPD pattern can be species specific (Guthrie et al., 1992) or can differentiate between various RAPD genotypes within a species (Hamelin et al., 1993) or even

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between strains (Bidouchka et al., 1994). If a RAPD pattern is coupled to a phenotypic trait, e.g. production of secondary metabolites, proteolytic activity, lipolytic activity or growth characteristics, the RAPD pattern can be used as a genetic marker. We used three primers (ari1, nor1 and omt1) for the RAPD analysis of 76 *P. roqueforti* strains used for blue cheese production, and tried to correlate the resulting RAPD groupings to phenotypic traits, e.g. production of secondary metabolites like PR-toxin, mycophenolic acid and isofumigaclavin.

2. Materials and methods

2.1. Strains and culture conditions

Seventy-six strains of *P. roqueforti* were analysed for their RAPD pattern, all from the culture collection of the Royal Veterinary and Agricultural University, Frederiksberg C, Denmark. They were isolated either from blue cheeses or obtained from suppliers of fungal starter cultures and maintained as described in Larsen et al. (1998). The strains were grown in malt extract medium (Merck, Darmstadt, Germany) under shaking conditions (180 rpm) at 25°C.

2.2. Isolation of fungal DNA

The isolation of DNA from fungal strains was performed according to a modified method originally described by Yelton et al. (1984). For that purpose, 72–96 h old mycelium was harvested from a submerged culture by filtration. The mycelium was transferred to a mortar and frozen in liquid nitrogen, ground in a mortar and resuspended in lysis buffer [50 mM EDTA, 0.2% SDS, pH 8.5 (Serva, Heidelberg)]. This suspension was heated to 68°C for 15 min and centrifuged for 15 min at 15,000 × *g*. After

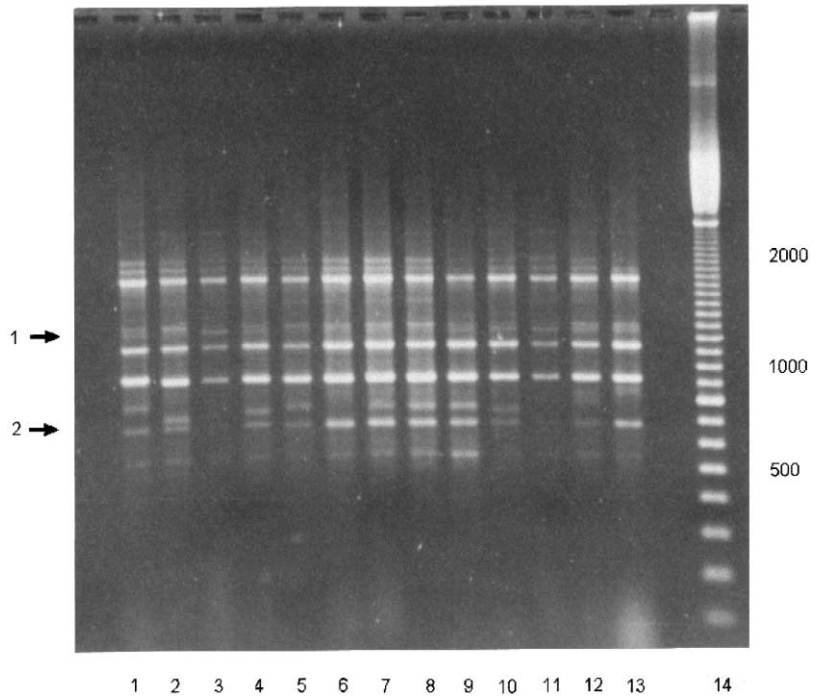

Fig. 1. Agarose gel of RAPD–PCR-products with chromosomal DNA of different *P. roqueforti* strains as template and with primer ari1. The two polymorphisms are marked with arrows. Lanes 1–13: *P. roqueforti* 32, *P. roqueforti* 47, *P. roqueforti* 20, *P. roqueforti* 52, *P. roqueforti* 69, *P. roqueforti* Fr4, *P. roqueforti* Fr6, *P. roqueforti* Fr7, *P. roqueforti* 63, *P. roqueforti* 50, *P. roqueforti* 10, *P. roqueforti* 11, *P. roqueforti* 18; lane 14: size marker (100 bp ladder, Pharmacia, Uppsala).

centrifugation, a volume of the supernatant (7 ml) was transferred to a new centrifuge tube and sodium acetate (4 M, 1 ml) added. This solution was placed on ice for 1 h and centrifuged for 15 min at 15,000 $\times g$. After centrifugation, the supernatant (6 ml) was transferred to a fresh tube, the solution extracted with phenol and the isolated DNA precipitated by the addition of ethanol (2.5 volumes). The isolated DNA was checked on an agarose gel and the concentrations were determined spectrophotometrically as described by Sambrook et al. (1989).

2.3. Polymerase chain reaction

The isolated chromosomal DNA was diluted to 2 $\mu g\ ml^{-1}$ and used as template DNA for RAPD–PCR reactions. A typical PCR reaction mixture contained: 5.0 μl Taq polymerase buffer (10 $\times$), 8 μl nu-

Table 1
Groupings of the *P. roqueforti* strains according to RAPD analysis with different primers and their production of mycophenolic acid[a]

	+ Mycophenolic acid	No mycophenolic acid	Not tested[b]
Primer ari1			
Group 1	10, Fr4		
Group 2	18, 19, 42, Fr5	41	
Group 3	1, 2, 9, 11, 40, 72	15, 35, 43, 73	61
Group 4		4, 32, 33, 37, 44, 46, 50, 53, 55, 57, 58, 71, 74, 75, 78, Fr2, Fr3, Fr6, Fr7, Mauri, CSL	31, 51, 60, 63, 70
Group 5		52	Pr
Group 6	12, 17, 34		
Group 7			79
Group 8	6, 13, 38		16, 76
Group 9		69	
Group 10	47		
Group 11	20	39, 45, 64	31
Group 12		54	
Group 13	21	36	5, 5.4, 5.5, 48
Primer nor1			
Group 1	21, 49, 67	36, 39, 41, 53, 57, 64, 68, Fr2	5.5, Fr1
Group 2	17, 40, 47, 66	4, 32, 33, 35, 37, 43, 44, 45, 46, 52, 54, 55, 58, 69, 71, 73, 74, 75, 78, Fr2, Fr3, Fr6, Fr7	5, 5.4, 31, 48, 51, 59, 63, 65, 70, 76
Group 3	20, 77		Chr
Group 4	1, 2, 9, 10, 11, 18, 19, 34, 38, 42, 72	7, 15	13, 16, 28, 60, 61, 79
Primer omt1			
Group 1	2, 6, 9, 10, 11, 12, 17, 18, 19, 20, 34, 38, 40, 42, 47, 49, 66, 67, 72, Fr4, Fr5	7, 15, 41, Mauri	13, 16, 28, 51, 59, 60, 61, 65, 79, Chr, Pr
Group 2	1, 21	4, 33, 35, 36, 37, 39, 44, 45, 46, 52, 53, 54, 55, 57, 64, 68, 69, 71, 73, 74, 75, 78, Fr2, Fr6, Fr7, CSL	5, 5.4, 5.5, 31, 48, 56, 63, 70, Fr1
Group 3		32, 50, 58	

[a]As the DNA of some strains did not give a RAPD pattern, not all the analysed 76 strains are listed for each primer. The numbers represent the strain numbers of the culture collection of the Royal Veterinary and Agricultural University, Frederiksberg, Denmark.
[b]Not tested for production of secondary metabolites.

cleotide mixture (dATP, dCTP, dTTP, dGTP; 2.5 mM each (Boehringer, Mannheim)), 4.0 μl $MgCl_2$ (25 mM), 1.25 μl primer (120 pmol μl^{-1}), 0.2 μl Taq polymerase (5 U μl^{-1} (Boehringer)), 5.0 μl template DNA (2 $\mu g\ ml^{-1}$), and 25.0 μl H_2O. Polymerase chain reactions were performed in 42 cycles (PerkinElmer GeneAmp 2400): 1 min at 95°C, 3 min at 36°C, 4 min at 72°C. The sequences of the random primers were as follows:

ari1,
5′CTGCTTGGCACAGTTGGCTTC3′
nor1,
5′ACCGCTACGCCGGCACTCTCGGCAC3′
omt1,
5′GTGGACGGACCTAGTCCGACATCAC3′

A specific feature of these primers are their lengths, 20–25 nucleotides rather than the 10 nucleotides usual for most described RAPD primers (Guthrie et al., 1992; Durand et al., 1993). An advantage of the longer primers is the much higher reproducibility of the PCR results.

2.4. Production and analysis of secondary metabolites

A total of 65 of the 76 isolates analysed for their RAPD patterns were also screened for production of secondary metabolites. Inoculation were done in three point cultures on yeast extract sucrose agar (YES) and the plates were incubated for 7 days at 25°C in the dark (for media formulation, see Samson et al., 1995). Extraction of the secondary metabolites and the subsequent HPLC analysis were performed according to Smedsgaard (1997).

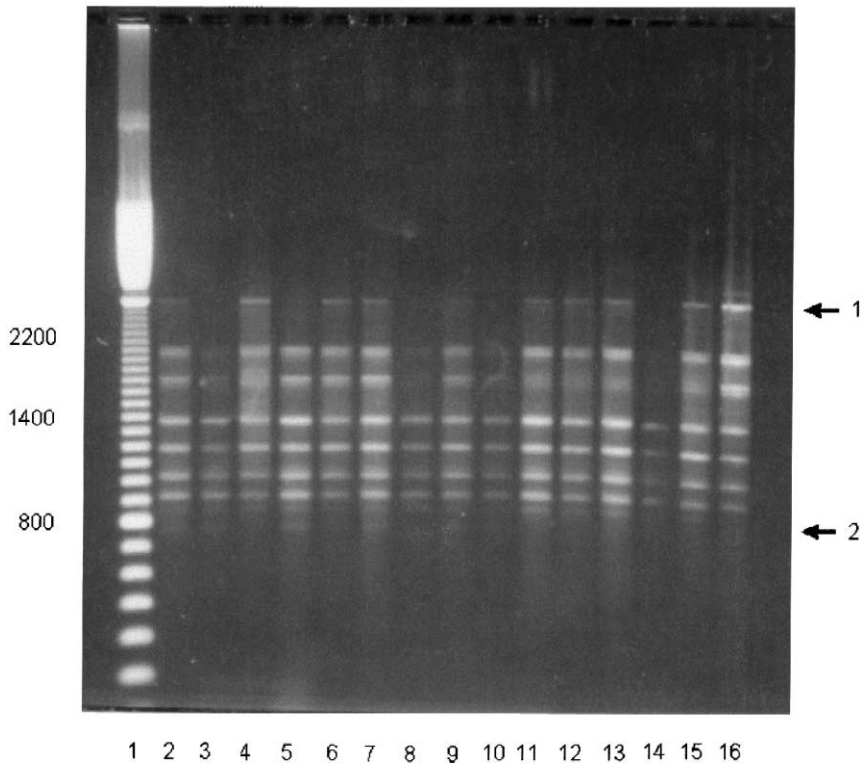

Fig. 2. Agarose gel of RAPD–PCR-products with chromosomal DNA of different *P. roqueforti* strains as template and with primer nor1. Fragment lengths are indicated in base pairs. Lane 1: size marker (100 bp ladder, Pharmacia, Uppsala); lanes 2–16: *P. roqueforti* 4, *P. roqueforti* 36, *P. roqueforti* 5, *P. roqueforti* 20, *P. roqueforti* 52, *P. roqueforti* 72, *P. roqueforti* 49, *P. roqueforti* 50, *P. roqueforti* 67, *P. roqueforti* 58, *P. roqueforti* 59, *P. roqueforti* 66, *P. roqueforti* 68, *P. roqueforti* 73, *P. roqueforti* 75.

3. Results

3.1. RAPD analysis of P. roqueforti strains

The patterns obtained by a particular primer were, in general, very similar, indicating high genotypic relatedness between the strains. However, because of micropolymorphisms within the pattern of each primer, the strains could be separated into different groups. As primers with a length of 21 and 25 nucleotides have been used, the RAPD pattern and also the micropolymorphisms were very reproducible.

With the primer ari1 PCR products between 2000 and 600 bp could be identified. This primer separated the strains into 13 groups based on two micropolymorphisms. In Fig. 1, polymorphism 1 is seen occurring in three forms: as a double band or one of the single bands (arrow 1), and polymorphism 2 in multiple forms: no band or bands with different fragment lengths (arrow 2). Table 1 summarizes the results of this analysis.

The RAPD patterns of the different *P. roqueforti* strains with the primer nor1 again are very similar between the strains. RAPD products with fragment lengths between 2500 and 800 bp appeared. According to some polymorphisms, the strains could be grouped into four groups. The polymorphisms were based on the presence or absence of two PCR products as seen in Fig. 2: a band with a length of about 2500 bp (arrow 1) and a band with a length of about 800 bp (arrow 2). The groupings of the strains are depicted in Table 1.

Primer omt1 was able to differentiate the strains into three groups. With this primer the strains showed similar but complex patterns. Many RAPD products

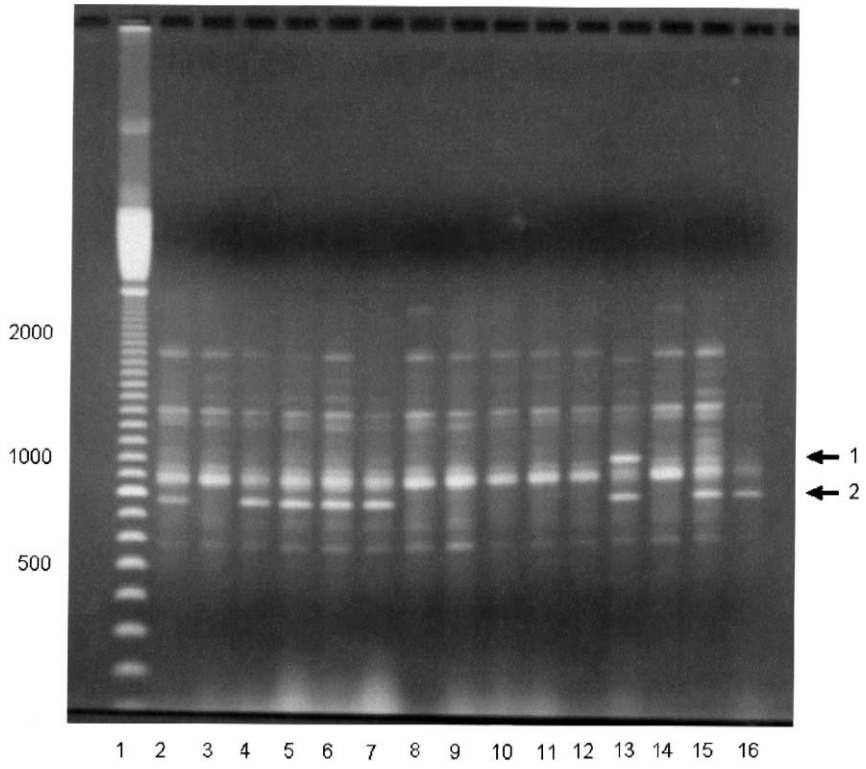

Fig. 3. Agarose gel of RAPD–PCR-products with chromosomal DNA of different *P. roqueforti* strains as template and with primer omt1. Fragment lengths are indicated in base pairs. Lane 1: size marker (100 bp ladder, Pharmacia, Uppsala); lanes 2–16: *P. roqueforti* 4, *P. roqueforti* 2, *P. roqueforti* 5, *P. roqueforti* 35, *P. roqueforti* 37, *P. roqueforti* 53, *P. roqueforti* 41, *P. roqueforti* 15, *P. roqueforti* 38, *P. roqueforti* 13, *P. roqueforti* 40, *P. roqueforti* 32, *P. roqueforti* 72, *P. roqueforti* 69, *P. roqueforti* 70.

Table 2
Production of secondary metabolites by strains of *P. roqueforti* from cheese origin

Strains	PR toxin	Roquefortine C	Mycophenolic acid	Isofumigaclavine A	RAI[a]	CAN[a]
4*	x + d[b]	x	–	–	–	x
7	x + d	x	–	x	–	x
15	x	x	–	–	–	x
32*	x	–	–	x	–	x
33*	d	x	–	x	x	x
35	d	x	–	–	–	x
36	x + d	x	–	–	–	x
37*	x + d	x	–	–	–	x
39	d	x	–	x	x	x
41	x + d	x	–	x	–	x
43	x + d	x	–	x	–	x
44*	x + d	x	–	x	x	x
45	x + d	x	–	x	x	x
46*	x	x	–	x	–	x
50*	x	–	–	x	–	x
52	x + d	x	–	–	–	x
53*	x + d	x	–	x	x	x
54	x + d	x	–	x	x	x
55*	x + d	x	–	x	x	x
57*	x + d	–	–	–	x	x
58*	x + d	x	–	–	–	x
64	x + d	x	–	–	x	x
68	x	x	–	x	–	x
69	x + d	x	–	–	–	x
71*	x + d	x	–	–	x	–
73	x + d	x	–	–	x	x
74*	x + d	x	–	–	–	x
75*	x + d	x	–	–	–	–
78*	x + d	x	–	–	–	x
PV	x + d	x	–	–	–	x
CSL*	x + d	x	–	–	–	x
Mauri*	x + d	x	–	–	–	x
FR2*	x + d	x	–	–	–	x
FR3*	x + d	x	–	x	–	x
FR6*	x + d	x + d	–	–	–	–
FR7*	x + d	x	–	x	x	x
1	x	x	x	x	x	x
2	x	x	x	–	x	x
3	x	x	x	x	x	x
6	x	x	x	x	x	x
8	x	x	x	x	x	x
9	x + d	x	x	x	x	x
10	x + d	x	x	x	x	x
11	x + d	x	x	–	–	–
12	x + d	x	x	x	–	–
13	x	x	x	x	x	x
14	x + d	x	x	x	x	x
17	x	–	x	x	x	–
18	x	x	x	x	x	x
19	x	x	x	x	x	x
20	x	x	x	x	x	x
21	x	x	x	x	x	x
34	x + d	x	x	x	x	x

Table 2 (*continued*)

Strains	PR toxin	Roquefortine C	Mycophenolic acid	Isofumigaclavine A	RAI[a]	CAN[a]
38	x	−	x	x	x	x
40	x	x	x	x	x	x
42	x	x	x	x	x	x
47	x	+	x	x	x	x
49	−	+	x	x	x	x
66	−	d	x	−	x	−
67	x	d	x	−	x	x
72	x	d	x	−	x	x
77	x	d	x	−	x	x
PJ	x	d	x	−	x	x
FR4	x + d	d	x	−	x	x
FR5	x	d	x	−	x	−

[a] The structure of RAI and CAN have not been determined.
[b] d: a derivative of the metabolite is produced.
[*] These strains belong to RAPD group 4 of primer ari1.

with fragment lengths between 2000 and 600 bp appeared. In contrast to the two previous primers, the grouping with this primer was based on more significant changes in the pattern (Fig. 3). In one group, a strong additional band of about 750 bp appeared (arrow 2), whereas in another group in addition to this band, a second band of similar strength with a fragment length of about 1000 bp appeared (arrow 1).

3.2. Correlation between the RAPD groups obtained with different primers

Different primers in general record the presence of different genetic polymorphisms, which should theoretically be independent. Table 1 shows a comparison of the grouping with the three different primers. From these results, it became obvious that the strains of a particular group of one primer are not randomly distributed over the groups from another primer. This is true for example for the strains from group 4, primer nor1. A total of 18 of the 19 strains from this group can be found in group 1, primer omt1. A similar behaviour can be observed with group 2, primer nor1. This group is very large consisting of 37 strains. Twenty-five of these strains, which means nearly 70%, can be found in group 2, primer omt1, and an even higher percentage of these strains (77%) can be found in group 4, primer ari1.

3.3. Correlation of the RAPD grouping with secondary metabolite production

Table 2 shows the secondary metabolites detected in 65 of the cheese isolates. All the strains tested produced PR-toxin or its derivates, apart from strains 49 and 66. Roquefortine C and its derivatives were also produced by the majority of the strains, 62 out of 65. The metabolite Can was detected in 86% of the strains whereas isofumigaclavine A and Rai both were present in about 55% of the strains. Only 45% of the strains produced mycophenolic acid.

The RAPD groups obtained with all three primers showed a correlation to the production of the secondary metabolites analysed. In all cases, the correlation were not absolute, but a clear tendency was seen. The 65 strains of *P. roqueforti* analysed for the production of secondary metabolites could be divided into two groups based on their production of mycophenolic acid. In Table 1, the RAPD grouping separates the strains producing mycophenolic acid from those that do not.

The following groups clustered mainly or only strains which did not produce mycophenolic acid: primer ari1, group 4; primer nor1, group 2; and primer omt1, group 2. The clusters primer nor1, group 4 and primer omt1, group 1 included mainly strains producing mycophenolic acid.

Another correlation could be observed with primer ari1. The group 4 of this primer apparently clustered

strains which have a lower capacity to produce secondary metabolites as most of the strains from this group produced less of the analysed metabolites compared to other strains (Table 2, strains with asterisks).

4. Discussion

Seventy-six strains of *P. roqueforti*, used as starter cultures for production of blue cheeses, were analysed by RAPD typing with the aim of analysing this technique for their ability to characterize these strains at a genotypic level and to find correlations between phenotypic and genotypic characteristics. The overall RAPD patterns were very similar for each primer for all the strains, which indicated that the analysed strains have a high degree of similarity at the genetic level. Nevertheless, by using the described primers slight genotypic differences between the strains could be observed and used for grouping the strains. The fact that the RAPD patterns of the starter strains are similar might be due to one of two possibilities. Either all the strains used as starters are clonal, and differences in genotype and phenotype have developed by mutational changes, or only strains with this genotype have the appropriate phenotype to be used as starters and have been selected for this purpose. Boysen et al. (1996) analysed different *P. roqueforti* strains by RAPD analysis. Their results are consistent with our results in that the RAPD patterns, which they found were also very homogenous within the analysed strains.

Despite the similarities in the RAPD patterns, it was possible to separate the analysed strains into different RAPD groups. Two of the primers only separated three or four groups, whereas one of them separated the strains into 13 groups. It was found that many of the groups produced by different primers were corresponding, e.g. most of the strains of a group from a particular primer could be found in one group of another primer instead of random distribution of the strains. From these results, it can be concluded that the observed different polymorphisms are not independent, but rather that occurrence of one polymorphism is coupled to the occurrence of a second polymorphism at high frequency.

A correlation between the RAPD patterns obtained and the production of secondary metabolites was observed. The production of mycophenolic acid for example corresponds well to the RAPD patterns obtained with all three primers. Group 4 of primer ari1 for example clusters only mycophenolic acid negative strains and also the other two primers generally separated the producing and non-producing strains. In addition group 4 of primer ari1 clustered strains which are less able to produce secondary metabolites than the other strains. This again is an indication that the detected polymorphisms are not independent, as all three polymorphisms can obviously be used as genetic markers for strains not producing mycophenolic acid.

The known secondary metabolites PR-toxin, roquefortine C, isofumigaclavine A and mycophenolic acid detected in this study have not been shown to pose a health risk to consumers, even though the last three metabolites mentioned have been found in blue cheese in small concentrations (Engel and Teuber, 1989). However, strains which would not produce these secondary metabolites would be favourable as starter cultures.

The results obtained here suggest that more extensive genotypic differences exist between strains producing mycophenolic acid and nonproducers than only the presence of the mycophenolic acid biosynthetic genes. In addition, one genotype discovered by RAPD grouping (primer ari1, group 4) obviously produces less secondary metabolites than other genotypes. These genetic differences might be useful for the selection of strains with reduced or impaired capacity to produce undesired secondary metabolites.

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