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Isolation and characterization of chlorate resistant mutants from nitrate-nonutilizing fungus *Phycomyces blakesleeanus*

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Chlorate resistant mutants, which were first isolated in the zygomycetous fungus *Phycomyces blakesleeanus*, were found to be resistant up to a concentration of at least 300 mM of potassium chlorate. The dose-response relationship showed that although the mutants could be divided into two groups based on chlorate resistance in the mycelial elongation assay on the solid minimal medium, this was not observed in the assay using liquid culture. Genetic analysis of heterokaryons revealed the mutant alleles to be dominant. Enzymatic activities of three nitrate reductases and chlorate reductase were deficient in both the parent strain and the mutants. Intracellular incorporation of chlorate ion varied from strain to strain; however, the variation could not explain the mechanism of chlorate resistance. One unexpected characteristic of the mutants was that the intracellular sulfate ion concentration was 3.5 to 5.5 times higher than in the parent strain. We designated this mutant genotype *crw*, chlorate resistant mutant from nitrate-nonutilizing wild type.

Key Words——chlorate resistance; dominant mutation; nitrate reductase; *Phycomyces blakesleeanus*, Zygomycetes.

Drug resistance in microorganisms is due to three major types of mechanisms. Type 1 is a modification in sequence or structure of the target proteins which hinders the binding of drug molecules or which prevents conformational changes of the target proteins in response to drug binding. This type of drug resistance is often phenotypically recessive as shown in streptomycin resistance (Foster, 1983). Type 2 is a cancellation of drug effects by direct or indirect counteraction of certain endogenic enzymes. As shown by the direct effect in the relation of aminoglycoside phosphotransferase to kanamycin, this type of resistance usually proves dominant (Davies and Smith, 1978). Type 3 is a change in the permeability of the plasma membrane, which does not allow the permeation of drug molecules into the cytoplasm (Davis and Maas, 1952).

In *Phycomyces blakesleeanus Burgeff*, several kinds of drug-resistant mutants have been isolated, including strains resistant to allyl alcohol (*adh*), 5-fluorouracil (*fur*), and 5-deazariboflavin (*dar*). Since the *adh*-mutants enzymatically lack alcohol dehydrogenase, they can be classified as Type 1 (Garcés et al., 1984). The analog 5-fluorouracil is incorporated into RNA as efficiently as uracil (Van Laere et al., 1976), leading to impaired protein synthesis. The *fur*-mutants were found to excrete uracil into a medium (Hilgenberg et al., 1987), and they can therefore grow with self-secreted uracil. This may be explained as being due to a competition between 5-fluorouracil and uracil which was increased in the medium by changed proteinous function (Type2). The *dar*-mutants showed a reduced ability to take up riboflavin and its analogs (Type 3) (Delbrück and Ootaki 1979).

Chlorate, a simple inorganic compound, is toxic to a number of organisms. Chlorate is not itself toxic, but is generally accepted, as first suggested by Åberg using young wheat plants in 1947, to be rendered toxic by conversion to chlorite as a result of the nitrate reductase-catalyzed reaction (see Cove, 1976). In fact, fungal mutant strains lacking nitrate reductase are also resisitant to chlorate in *Ustilago maydis* (de Candolle) Corda (Lewis and Fincham, 1970) and *Aspergillus nidulans* (Eidam) Winter (Cove, 1976). On the other hand, chlorate is a strong inhibitor of nitrate uptake in *A. nidulans*, the process of which is separate and distinct from, but dependent on, the nitrate reductase reaction (Brownlee and Arst Jr., 1983). Chlorate is thought to act as a nitrate analog (Cove, 1976; Griffin, 1994). During a search for a new resistance marker that might be useful for genetic transformation, we found that chlorate is also toxic for *Phycomyces* and that the mutants resistant to the drug show a dominant phenotype. Since *Phycomyces* possesses neither nitrate reductase nor the ability to utilize nitrate (Garcés et al., 1985), chlorate might be rendered toxic by reduction enzyme(s) other than nitrate reductase, or by another as yet unidentified mechanism. In the present paper, we describe the first isolation of chlorate resistant mutants from a nitrate-nonutilizing fungus and their characterization.

Materials and Methods

Strains and culture conditions *Phycomyces* strains used in this work are listed in Table 1. The wild mating type (-) of *P. blakesleeanus*, IGE1101 (=NRRL 1555), was used

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Table 1. Strains of *Phycomyces blakesleeanus* used in this work.

Strains	Genotypes (mating type)	Source (reference)
IGE1101	(−)	NRRL1555
IGE1103	(+)	A56, isogenic to NRRL1555 (Alvarez and Eslava, 1983)
C2	*carA5* (−)	From NRRL1555 (Meissner and Delbrück, 1968)
C5	*carB10 geo-10* (−)	From NRRL1555 (Isolated by M. Heisenberg)
S102	*nicA101* (−)	From NRRL1555 (Medina, 1977)
Y92	*crw-1 nicA101* (−)	From S102 in this work
Y93	*crw-2 nicA101* (−)	From S102 in this work
Y94	*crw-3 nicA101* (−)	From S102 in this work
Y95	*crw-4 nicA101* (−)	From S102 in this work
Y96	*crw-5 nicA101* (−)	From S102 in this work
Y97	*crw-6 nicA101* (−)	From S102 in this work
Y98	*crw-7 nicA101* (−)	From S102 in this work
Y99	*crw-8 nicA101* (−)	From S102 in this work

to estimate chlorate effects. Chlorate resistant mutants were induced from S102, a nicotinic acid-requiring auxotrophic mutant (genotype *nic*) derived from NRRL 1555. IGE1103 (=A56), which is a (+) wild type isogenic to NRRL 1555, was used as a mating partner to observe sexual reactions. C2 and C5, white color mutants due to the defect of β-carotene, were used for making heterokaryons with each of the chlorateresistant mutants. *Neurospora crassa* Shear & Dodge IFO 6966 was used as a positive control strain for the chlorate and nitrate reductase activities and the nitrogen utilization test.

The *Phycomyces* cultures were routinely grown at 20–22°C on solid SIV minimal medium (Sutter, 1975) or complete PDAYC medium (Cerdá-Olmedo, 1987; Yamazaki et al., 1996) supplemented appropriately. Constant irradiation by white fluorescent light (a 40 W lamp at a distance of 30 cm) was carried out without a mating reaction. To allow growth of the *nic* strain, nicotinic acid (10 μg/ml) was added to the medium (SIVnic). For the selection medium to isolate chloratere-sistant mutants, SIVnic medium containing 200 mM $KClO_3$ (SIVnic+$KClO_3$) was used. When compact colonies were required, TritonX-100 (final 0.02%, w/v) was added to the medium. For mating reactions, SI minimal medium (Sutter, 1975) was used. Liquid media contained the same ingredients as the respective solid media with the exception of agar. Liquid culture was continuously agitated in a figure-eight pattern (40 cycles/min). *Neurospora crassa* was cultured on Fries minimal medium (Ryan et al., 1943), the nitrogen source in which was changed to sodium nitrate for the chlorate and nitrate reductase assays.

Isolation of chlorate-resistant mutants Mutant selection was performed according to the method of Heisenberg and Cerdá-Olmedo (1968). After breaking the dormancy by heat shock (10 min at 48°C), the sporangiospores of S102 were treated for 30 min with 0.01% N-methyl-N-nitro-N-nitrosoguanidine (NTG) in 0.1 M citrate-phosphate buffer (pH 7.0) at a final concentration of 5×10^7 spores/ml. The spore suspension was incubated in the dark at room temperature with agitation (120 rpm). The spores collected by centrifugation (5 min, $2000\times g$) were then washed five times in distilled water. Approximately 4×10^5 spores per Petri dish (140 mm in diam) were inoculated on selection medium (SIVnic+$KClO_3$). After 7 d (first screening), a single colony growing well on a plate was picked up and transferred to fresh selection medium in a smaller dish (60 mm in diam). Sporangiospores of a single sporangium derived from this colony were collected, inoculated on the selection medium supplemented with TritonX-100 to form a compact colony, and cultured for 48 h. Then 10 colonies were transferred to the selection medium and cultured until the sporangia ripened. A single sporangium from a well-growing colony of the 10 colonies was again used for compact colony formation. To define a stable phenotype, we repeated this series of steps 8 times.

Heterokaryon analysis Heterokaryons were constructed by grafting decapitated young sporangiophores from two genetically different strains (Ootaki 1987). Each sporangium from the regenerant between C2 or C5 and the mutants was separately suspended in distilled water to make a spore suspension. The heterokaryotic spores were used to determine if the mutation was phenotypically dominant or recessive. When the spores are inoculated on SIV+$KClO_3$ medium, they must be able to germinate and develop mycelia if they are of dominant phenotype. As a technical control for successful heterokaryon formation, a part of the spores from C5*S102 was also inoculated on SIV medium on which heterokaryotic mycelia should appear yellowish. At least 5 sporangia from each combination were tested.

Enzyme assays Cell-free extracts were prepared by the method of Garrett and Cove (1976). Fresh mycelia were ground in a ice-cold mortar and pestle with a small amount of quartz sand in 4 to 5 ml of preparation buffer per gram of mycelia. The preparation buffer consisted of 0.1 M phosphate buffer (pH 6.8), plus 1 mM β-mercapto-ethanol, 0.5 mM EDTA, 1% (w/v) NaCl and 0.1% phenylmethyl sulfonyl fluoride. The crude homogenate was centrifuged at $25{,}000\times g$ for 20 min at 4°C, and the supernatant fraction was used for enzyme assays. The

chlorate reductase activity was assayed by iodometry, a method in which the blue color depending on the amount of chlorite with amylose-iodine complex is titrated with $Na_2S_2O_3$ till it turns clear (Pichinoty et al., 1969). The activity of nitrate reductase was determined spectrophotometrically by three assay methods: NADPH-nitrate reductase (Garrett and Cove, 1976; Nicholas and Nason 1957), $FADH_2$-nitrate reductase (Garrett and Nason, 1969), and methyl viologen-reduction by nitrate reductase (Garrett and Nason, 1969).

Ion chromatography Several pieces of mycelial mat of both S102 and chlorate resistant mutants were inoculated and cultured in 50 ml of SIVnic liquid medium for 4 days. The round mycelial colonies formed in the medium were then transferred to SIVnic or SIVnic containing 100 mM $KClO_3$ and incubated for 1 h. They were then placed on ashless filter paper in a Buchner funnel, quickly rinsed with distilled water, and drained by aspiration. After weighing fresh mycelial colonies, they were transferred to screw-top test tubes containing 2 ml of distilled, deionized water. Extraction was performed by boiling the tubes for 2 h. The broth collected was passed through to an ultrafiltration module (Molcut II LCC5000, Nihon Millipore Ltd., Tokyo). The eluent was used immediately for ion chromatography or stored at -20°C until use. Chlorate and other ions were measured using a flow path-switchable ion chromatography system equipped with a conductivity detector (ICA-5000, Toa Electronics, Tokyo) and a HPLC-packed column (PCI-201S for anion analysis and PCI-311 for cation analysis) (Sasaki et al., 1999). A mixture of 2.5 mM phthalate + 2.5 mM Tris (hydroxymethyl)aminomethane and 12 mM tartaric acid was used as elution solvent for anions and cations, respectively. Intracellular ion concentration was calculated by multiplying the coefficient values of the dilution and the cytoplasmic ratio.

Results

Effects of chlorate and chlorite on *P. blakesleeanus* wild type In the wild type, chlorate has no effect on the spore germination, its ratio of 85–90% being quite normal until 200 mM $KClO_3$. The hyphal elongation of germlings and the development of colonies, however, were seriously retarded. The toxic effect of chlorate on *P. blakesleeanus* wild type (IGE1101) was determined by measuring the mycelial elongation on chlorate-supplemented SIV media (Fig. 1). Growth inhibition was already manifested at a concentration of 50 mM $KClO_3$, where elongation was reduced to 16% of the control. The radius of mycelia was 15% that of the control even at a concentration of 200 mM $KClO_3$. From a concentration of 300 mM upwards, no growth occurred. NaCl and KCl, which were used to check the effect of osmotic stress, both reduced the elongation to around 55% of the control at concentrations of 50 to 300 mM, and mycelial growth was completely inhibited only at a very high concentration of 800 mM. From these results, we conclude that 50% in the inhibition of mycelial elongation induced by chlorate was not caused by the elevation of osmotic

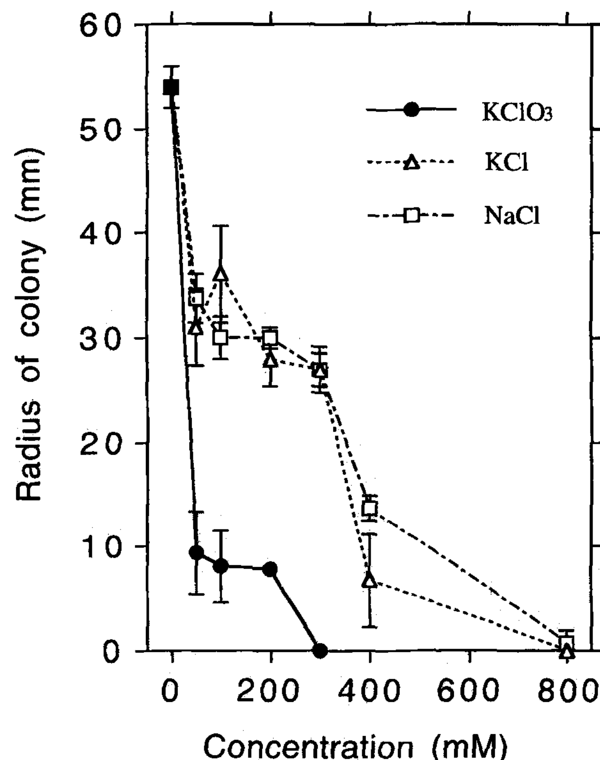

Fig. 1. Effect of various concentrations of potassium chlorate, potassium chloride and sodium chloride on mycelial growth in wild-type *Phycomyces blakesleeanus*, IGE1101. These chemicals were added to SIV minimal medium. Mycelial front (3×3 mm) was inoculated on the edge of the culture dish and the radius of colony was measured after a 5-d culture at 20–22°C. The mean radius with standard deviation for three colonies is expressed in each plot.

pressure in the presence of these salts, but mainly by some specifically toxic effect. On the other hand, the wild type did not form any visible colony on the SIV medium containing only 1–2 mM chlorite.

Isolation of chlorate-resistant mutants Strain S102, which is an auxotroph for nicotinic acid derived from the wild type NRRL1555, was mutagenized as described in Materials and Methods. The viability of the NTG-treated spores was 26%. A total of 5×10^7 spores was subjected to selection on SIVnic+$KClO_3$ medium. In the first screening after 7 d, 53 primary candidates were chosen, transferred to fresh selection medium, and cultured until the sporangiospores ripened. Since *Phycomyces* is a multi-nucleate organism, in order to obtain stable phenotypes, the asexual sporangiospores were passed through eight sequential steps for unification of the nuclear composition. Eight stable mutants resistant to chlorate were finally obtained (Table 1). A typical strain resistant to chlorate, Y98, is shown in Fig. 2. Y98 displayed growth supported by thick mycelia on which sporangiophores, each with a sporangium, developed normally. All the other mutants showed the same developmental process, except that Y96 formed thin sporangiophores. The effect of KCl on Y98 was observed to be of the same extent as that of S102. NaCl

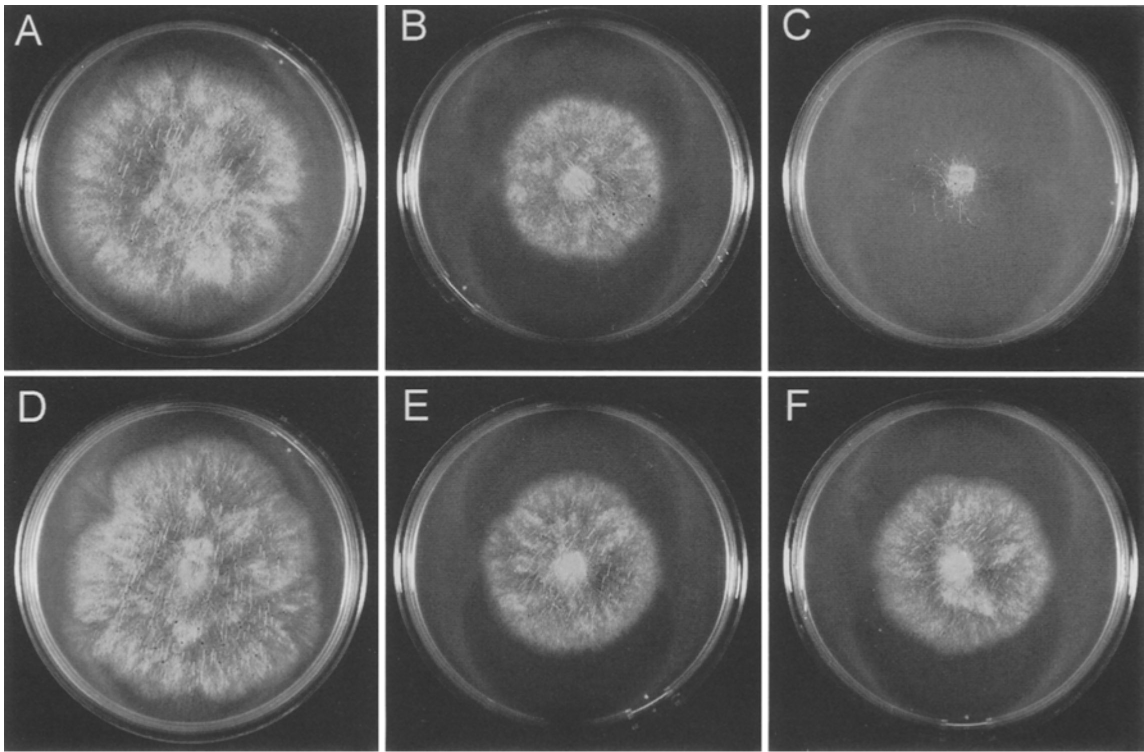

Fig. 2. Growth of the parent strain S102 and its chlorate resistant mutant Y98.
S102 (A, B and C) and Y98 (D, E and F) were grown for 4 d on SIVnic (A and D), SIVnic+200 mM KC1 (B and E), and SIVnic+200 mM $KClO_3$ (C and F). Note that although mycelia of S102 elongates by an extent similar to those of Y98 even in the presence of 200 mM $KClO_3$, the mycelia show very poor spreading.

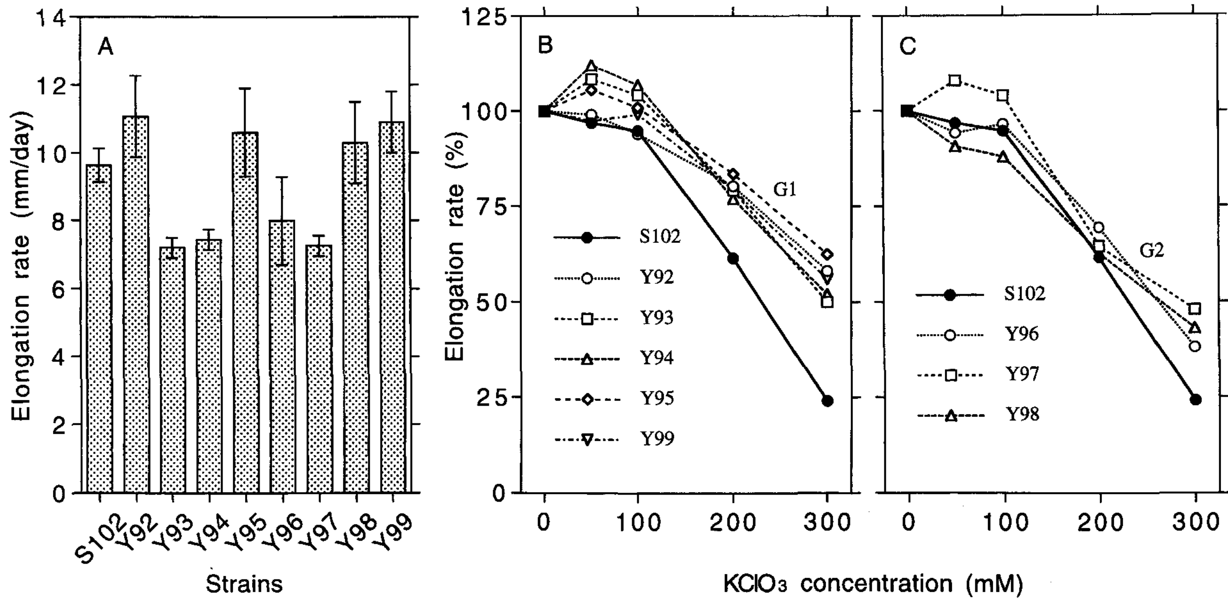

Fig. 3. Growth analysis of chlorate resistant mutants on solid medium.
A. All the 8 mutants and S102 as a control were cultured at 20–22°C on SIVnic medium without chlorate. The mean elongation rates with standard deviation were calculated for a period of 5–7 d. B, C. Dose-response curves of potassium chlorate for strains divided into two arbitrary groups, G1 (B) and G2 (C). Each value (elongation rate) along an elongation line was normalized to the value without chlorate as 100%. All values are from at least 3 separate experiments.

had exactly the same effect (data not shown). The same osmotic effect was also observed in two other mutants, Y95 and Y99. This indicates that the response to osmotic stress is not affected by the mutation. Note that although mycelia of S102 elongated to an extent similar to that of Y98 even in the presence of 200 mM $KClO_3$, the mycelia showed very poor spreading (Fig. 2). Sexual reaction of the mutants was examined on SI medium at 20°C in the dark. Four strains, Y92, Y95, Y98, and Y99, showing growth equivalent to the recipient S102, each manifested a normal mating reaction, resulting in the formation of mature zygospores.

Growth analysis of resistance Dose-response relationship was examined by measuring the elongation rate of cultures grown on the media containing different concentrations of chlorate (Fig. 3). As the elongation rate on the medium without chlorate was different among the mutants (Fig. 3A), the response (elongation rate) parameter is shown as a percentage of the elongation rate without chlorate in each strain (Figs. 3B, 3C). First, sensitivity of the parent strain S102 was quite different from that of the wild-type strain IGE1101 in the experiment in which mycelial elongation was measured (Figs. 1, 3). Even at 100 mM $KClO_3$, S102 showed a plausible growth of 93%. It apparently seemed to mask the phenotype resistant to chlorate in the mutants. The elongation of S102 was, however, supported by the poor, sparse hypha as shown in Fig. 2. To confirm the real resistance, we measured the growth mass yielded by liquid culture. In this experiment, by direct comparison, we selected the strains with growth equivalent to S102. As the result, the growth of S102 was reduced almost linearly until 200 mM, at which no mass increase was observed (Fig. 4). In addition to this expected behavior, the mutants clearly showed resistance to chlorate, judging from the curve which is similar to that in Fig. 3 (Fig. 4). It was apparently possible to divide mutants into two groups, G1 and G2, based on their resistance shown in the dose-response curves in the elongation assay (Figs. 3B, 3C), but this was not the case in the assay using liquid culture (Fig. 4). As the mutants showing different elongation rates were included in the same group, the additional phenotype in which the elongation rate was reduced was thought to be an individual phenomenon, or another mutation occurring simultaneously. Further experiments were, therefore, carried out using the strains Y92, Y95, Y98, and Y99, which show growth similar to that of the parent S102.

Heterokaryon analysis Heterokaryon analysis using the grafting method was performed to determine whether the phenotype of chlorate resistance is dominant or recessive. The mutants have three phenotypical characteristics, namely yellow-color colonies, auxotrophy for nicotinic acid, and resistance to chlorate. The partner strain C5 is an albino, not requiring nicotinic acid and sensitive to chlorate. When the sporangiospores from a sporangium derived from the sporangiophore regenerated at the point of grafting were collected and inoculated on the medium of SIV+$KClO_3$, a mycelial mat showing resistance to chlorate with yellowish-color appeared in, for example, the combination between C5 and Y92 (Fig. 5B). In all the other combinations, the same phenomenon was observed, although the formation efficiency of

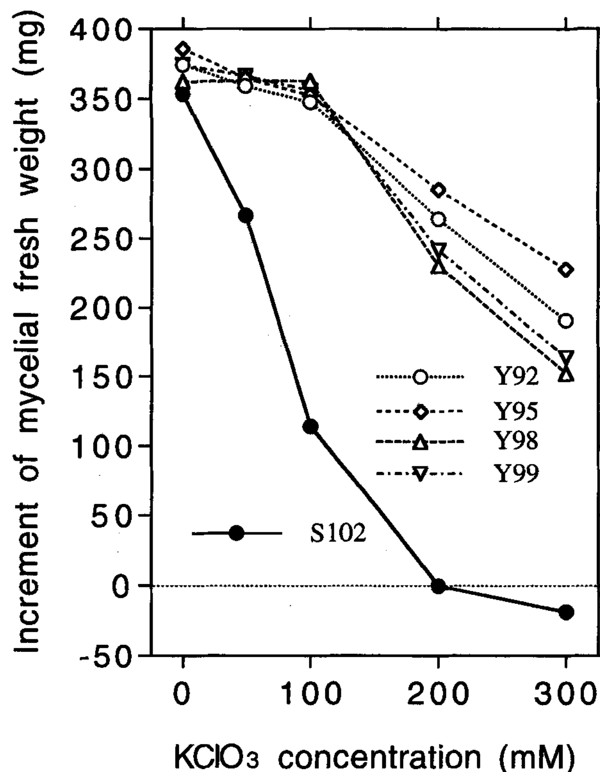

Fig. 4. Growth analysis of chlorate resistant mutants in liquid medium.
Small pieces of mycelia from typical mutant strains were weighed before inoculation, then cultured in SIVnic+$KClO_3$ medium for 4 d. Each value shows the mean from 2 separate experiments.

Table 2. Chlorate resistance of the mycelia derived from heterokaryotic spores of the sporangia via sporangiophore-grafting.

Heterokaryon	n_i[a]	n_c[b]	$n_c/n_i \times 100$
C5*S102	7[c]	3[c]	43[c]
C5*Y92	6	2	33
C5*Y95	6	4	67
C5*Y98	6	3	50
C5*Y99	5	4	80

a) The initial number of sporangia via sporangiophore-grafting, the spores of which were used for the test of chlorate resistance.
b) The number of sporangia via sporangiophore-grafting, the spores of which germinated and spread mycelia (yellowish color) on SIV plates containing $KClO_3$, except in the case of C5*S102.
c) As a control, the initial number (7) of sporangia from C5*S102, the spores of which were spread on SIV plates. The spores from 3 out of the initial 7 sporangia spread yellowish mycelia, indicating that the 3 sporangia (43%) contained heterokaryotic spores.

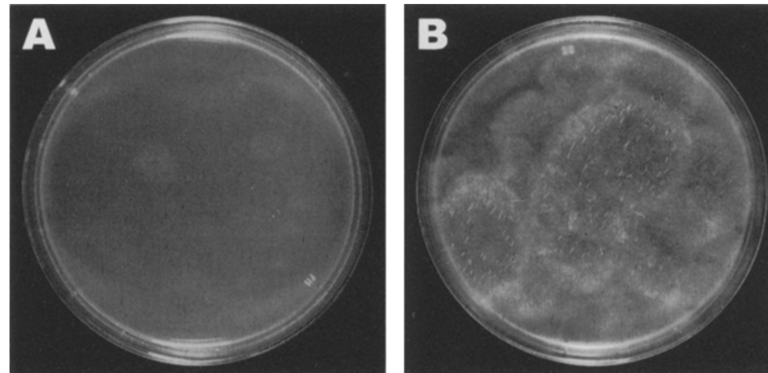

Fig. 5. Dominance of chlorate resistant mutants in heterokaryons.
Spores from the heterokaryon C5*S102 (A) as a negative control and C5*Y98 (B) were inoculated and cultured for 4 d on SIV+$KClO_3$ medium.

real heterokaryons varied from 33 to 80% (Table 2). Moreover, the heterokaryotic sporangiospores from the mycelial mats germinated well and again produced many sporangiophores on the medium SIV+$KClO_3$ (data not shown). On the other hand, several poor colonies also appeared in the control combination, C5*S102 (Fig. 5A), and developed poor sporangiophores, the spores of which did not produce any colonies. These findings indicate that all the yellowish mycelial mats evolved from the real heterokaryons, and that the chlorate resistance was dominant.

Enzymatic analysis To obtain information on the chlorate resistance in *Phycomyces*, the activities of both chlorate reductase and nitrate reductase were assayed using S102 and the mutants. As *Phycomyces* could not grow in the medium containing nitrate as the sole nitrogen source, the mycelium grown in SIVnic (asparagine as a nitrogen source) was used for preparation of crude extract. The reduction of chlorate to chlorite by crude enzymes was quantified by iodine titration with $Na_2S_2O_3$ of amylose-iodine complex, the concentration of which was dependent on the chlorite content. Unfortunately, chlorate reductase was below the limit of detection in both S102 and the mutants as well as *N. crassa*, although the standard titration curve was linearly quantifiable up to 75 nmols, confirming published results (Pichinoty et al., 1969). Nitrate reductase activity was tested with three different assays: NADPH-, $FADH_2$-, and reduced methyl-viologen-dependent nitrate reductases. No significant activity indicating that *Phycomyces* possesses any of the three nitrate reductases was found in either S102 or the mutants, as also described in a previous paper (Garcés et al., 1985), although significant activity was detected in *N. crassa* grown in nitrate-containing medium.

Ion chromatography We measured the intracellular concentration of incorporated chlorate by ion chromatography. After 1 h of exposure in chloratecontaining medium, S102 incorporated chlorate to a level of 2.17±0.34 mM (Table 3). Incorporation by the mutants varied from strain to strain, from 1.85±0.21 to 3.59±0.36 mM (Table 3). This result indicates that chlorate resistance can not be explained by the difference in chlorate permeability between S102 and the mutants. In anion analysis, we detected chloride and sulfate ions in addition to the chlorate ion, concentrations of which were 0.34 and 2.21 mM in the fresh culture medium, respectively. No difference in chloride incorporation was observed between S102 and the mutants, but its level was almost doubled by chlorate exposure, from 0.93±0.14 mM (-chlorate) to 1.79±0.36 mM (+chlorate). Interestingly, although the intracellular sulfate ion of S102 was controlled at a lower level compared with the fresh medium, and a 3.5- to 5.5-fold higher concentration was observed in the mutants even without chlorate exposure (Table 3). It seemed that chlorate exposure affected only the base value (Table 3). In cation analysis, we detected sodium, ammonium, potassium, magnesium, and calcium ions, whose concentrations in the fresh medium were 0.064, 0.32, 32.20, 2.03, and 0.20 mM, respectively. Their levels of incorporation were 40-, 50-, 3-, 7-, and 70-fold, respectively. The tendency of incorporation was similar in both S102 and the mutants, regardless of the presence of chlorate (data not shown).

Table 3. Intracellular concentration of chlorate and sulfate ions as shown by ion chromatography.

Strains	Intracellular concentration (mM)[a]		
	ClO_3^-	SO_4^{2-}	
	+$KClO_3$	-$KClO_3$	+$KClO_3$
S102	2.17±0.34	0.71±0.13	0.92±0.28
Y92	1.85±0.21	3.92±0.29	4.52±0.89
Y95	3.59±0.36	2.80±0.18	3.63±0.26
Y98	2.22±0.32	3.39±0.50	3.95±0.54
Y99	3.34±0.68	3.22±0.61	4.38±0.86

a) Each figure shows mean±standard deviation from at least 3 separate measurements, carried out after 1 h-exposure to the medium with or without $KClO_3$.

Discussion

In this study, we first induced chlorate resistant mutants of the zygomycetous fungus *P. blakesleeanus* from S102, which is an auxotroph for nicotinic acid, with NTG. Since 4 strains out of 8 resistant mutants showed equivalence to the parent strain in elongation rate without chlorate stress, they were analyzed in detail. Due to the unexpected behavior of S102, the resistance was somewhat ambiguous in the mycelial elongation assay on solid medium. However, in the growth mass assay in liquid medium, we found that the mutants had a real resistance to chlorate up to at least 300 mM, at which S102 had a minus increment, implying complete death. From dose-response relationships in the elongation assay, it was apparently possible to divide mutants into two groups, G1 and G2, the former group showing relatively higher resistance than the latter. However, such grouping was not possible in the assay using liquid culture. Sensitivity to chlorate is known to vary with culture conditions such as nutrition or pH even in the same mutation group in *A. nidulans* (Cove, 1976). Water stress or semi-aerobic conditions may affect sensitivity to drugs. Characteristics of the mutant strains such as response to osmotic stress (200 mM KC1 or NaCl), completion of sporangiophore development, and normal zygospore formation through sexual reaction were almost the same as those of the parent strain, indicating that these are independent of chlorate resistance.

In nitrate-utilizing fungi, most of the chlorate resistance is acounted for by five mutations: *niaD*, a structural gene for nitrate reductase; *crnA*, a gene for nitrate permeation or incoporation; *cnx*, genes for molybdenum-containing co-factor; *nirA*, a positive-acting gene for nitrate induction; and *areA*, a positive-acting gene for nitrogen metabolite repression (Cove, 1976; Unkles et al., 1989). In a preliminary experiment, we expected that *Phycomyces* would be resistant to chlorate, because of the absence of functional nitrate reductase and the nitrate-nonutilizing trait in the wild type (Garcés, 1985; this report). The result, however, was the opposite, suggesting that another process or mechanism underlies this phenomenon. Despite many efforts, including assays of enzymes that might be related to chlorate metabolism and nitrogen utilization tests, we were unable to obtain concrete evidence of the cause of chlorate resistance in this report, implying that the resistance cannot be explained as being Type 1. We therefore chose to designate this mutant genotype *crw*, chlorate resistant mutant from nitrate-nonutilizing wild type.

Chlorate resistance in *Spirulina platensis* has been suggested to be due to the loss of permeability to chlorate (Lanfaloni et al., 1994). To investigate this possibility, we attempted to apply ion chromatography to measure chlorate incorporated during a period of one hour, sufficiently limited to avoid a secondary effect of damage due to long exposure. Measurement of intracellular ion concentrations was carried out in *Phycomyces* for the first time. The result indicated that chlorate resistance could not be explained by the difference between S102 and the mutants in chlorate permeability, and thus that the mutants do not operate by the Type 3 mechanism.

We found that S102 and the mutants differ in the amount of intracellular sulfate ion. Even without chlorate stress, the level of the sulfate ion was 3.5- to 5.5-fold higher in the mutants than in S102. It is known that *Cunninghamella elegans* (Lendner) Lunn & Shipton, a zygomycetous fungus, has the potential to detoxify or inactivate various polycyclic aromatic hydrocarbons by forming sulfate conjugates (Pothuluri et al., 1996; 1998). Other zygomycetous fungi, *Mucor rouxii* (Calmette) Wehmer and *Mortierella isabellina* Oudemans, have a similar metabolic system (Moussa et al., 1997), which may operate in an emergency via sulfate utilization in Zygomycetes. In *Phycomyces*, the high intracellular sulfate maintained in the mutants might be utilized for rescue or inactivation of chlorate-associated effects, since sufficient chlorite to inhibit growth could not be formed in this fungus. If the proteinous function responsible for sulfate transport changes for emergency, the resistance mechanism may be Type 2.

From heterokaryon analysis, we found that the chlorate resistance was dominant. In this analysis we first used the C2 strain as a partner for the chlorate-resistant mutants. Unfortunately, C2 expressed the same character as S102, leading to ambiguous results in the analysis. Since C5 has the same sensitivity to chlorate as the wild-type IGE1101, clear data were obtained with the heterokaryons between C5 and the mutants. There is now no information as to whether the relatively significant resistance to chlorate accompanies *nicA* and *carA* on S102 and C2, respectively. On the other hand, chlorate resistant mutants, which are both *nit1* (identical to *niaD*) and *nitM* (identical to one of *cnx* genes) in *Glomerella graminicola* Politis (Vaillancourt and Hanau, 1994) and *Fusarium poae* (Peck) Wollenweber (Liu and Sundheim, 1996), are recessive, as shown by a complementary heterokaryon test. In *Phycomyces*, only one mutant so far studied has been isolated that is resistant to 5-fluorouracil (Alvarez et al., 1980), showing a nucleous inherited dominant mutation. This resistance may be explained as being due to the Type 2 mechanism described in the Introduction (Hilgenberg et al., 1987).

It has been pointed out that the inability to utilize nitrates is common among the higher Basidiomycetes, the Saprolegniaceae, the Blastcladiales, and certain members of the Mucorales (Whitaker, 1976). This suggests that the capacity to use nitrate can be lost by mutation and that survival of strains harboring this mutation is probably governed by their ecological situation rather than their taxonomic position (Whitaker, 1976). According to our knowledge, no isolation is reported on mutants resistant to chlorate in these groups. The *crw* mutant obtained in this study may provide new insights for understanding the ecological and physiological situations regarding the difference between nitrate-utilizing and -nonutilizing fungi.

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