Chlorate-resistant, Nitrate-utilizing (crn) Mutants of Fusarium moniliforme (Gibberella fujikuroi)

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Five crn loci have been identified in Fusarium moniliforme. Mutants at these loci were chlorate resistant and able to utilize nitrate. The crnl locus was closely linked to, and may be allelic with, nit3, a regulatory gene controlling induction of the nitrate reductase pathway. The crn2 locus was linked to, but distinct from, the nit5 gene coding for a molybdenum-containing cofactor. The crn3 and crn4 loci were unlinked to any known gene affecting nitrate reduction. The crn5 locus was clearly linked to the nitl gene coding for the nitrate reductase enzyme. Mutants of crnl and crn5 had low but detectable levels of nitrate reductase activity; these mutations may be leaky alleles of nitrate reduction genes. crn2 mutants had normal nitrate reductase activity, and may be altered in nitrate uptake.

INTRODUCTION

Fusarium moniliforme (Sheld.) emend. Snyd. and Hans. [sexual stage Gibberella fujikuroi (Sawada) Wr.] is a filamentous ascomycete which can produce carcinogenic mycotoxins (Gelderblom et al., 1988) and cause stalk rot of maize and sorghum and equine leukoencephalomalacia of horses and donkeys (Nelson et al., 1981; Marasas et al., 1984). The fungus is heterothallic and can be readily crossed in the laboratory. Strains of F. moniliforme mutate spontaneously on medium containing chlorate, producing fast-growing, chlorate-resistant sectors (Puhalla & Spieth, 1985; Klittich et al., 1988; Klittich & Leslie, 1988). Chlorate is a toxic analogue of nitrate, and most of the sectors consist of mutants which cannot utilize nitrate (nit mutants). Some sectors, however, are both chlorate resistant and able to utilize nitrate (CRUN sectors) (Klittich & Leslie, 1987, 1988).

Chlorate-resistant, nitrate-utilizing (crn) mutants have been found in Aspergillus nidulans, Septoria nodorum and Fusarium oxysporum (Cove, 1976b; Tomsett & Cove, 1979; Newton & Caten, 1988; Correll et al., 1987). Cove (1976b) suggested that mutations in at least three loci in A. nidulans could result in a CRUN phenotype, but only one locus, crnA, has been described (Tomsett & Cove, 1979). The crnA locus is tightly linked to structural loci for nitrate reductase and nitrite reductase (Tomsett & Cove, 1979) and appears to be involved in nitrate uptake (Brownlee & Arst, 1983).

The purposes of this study were to determine if stable crn mutants are produced by F. moniliforme, how the mutations are inherited, and the effect of the mutations on nitrate metabolism. We determined the composition of CRUN sectors, identified mutants with CRUN phenotypes, mapped crn mutants relative to other loci affecting nitrate metabolism, and examined the effect of crn mutations on nitrate reductase activity. Preliminary results were reported previously (Klittich & Leslie, 1987).

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Abbreviation: CRUN sectors, chlorate-resistant sectors that can utilize nitrate.
**Characterization of sectors.** Chlorate-resistant sectors were isolated and tested for nitrate utilization in a previous study (Klittich & Leslie, 1988). Most sectors (68–92%, depending on the strain) contained nit mutants and could not metabolize nitrate. Some sectors, however, were both chlorate resistant and able to utilize nitrate (CRUN sectors). Forty CRUN sectors from strains 102, 124, 133, 171, 409 and 410 were characterized by culturing 10–30
chlorate resistance and nitrate utilization. In all experiments, cultures were incubated at 25 °C with a 12 h light/12 h dark cycle. nit mutants obtained from CRUN sectors were sorted into phenotypic classes according to their utilization of five different nitrogen sources (Correll et al., 1987). Mutants which could utilize ammonium, nitrite and hypoxanthine, but not nitrate, had a Nit1 phenotype; strains which could utilize neither nitrate nor nitrite had a Nit3 phenotype; and strains which could utilize neither nitrate nor hypoxanthine had a NitM phenotype.

**Genetic analysis.** crn mutants were crossed to identify the number of loci giving a CRUN phenotype. Unique crn mutants were crossed with mutants at seven nit loci to determine linkage relationships with nitrate metabolism genes. Crosses were made on carrot agar and ascospores were isolated using a Cailloux stage-mounted micromanipulator as described previously (Klittich & Leslie, 1988). Progeny were transferred from complete medium to minimal chlorate medium and minimal medium (Correll et al., 1987) to test for resistance to chlorate and for the ability to utilize nitrate. The subcultures were scored for growth 4 d after transfer. Progeny with ambiguous growth patterns were also scored 7 d after transfer. Genetic terminology follows guidelines of Yoder et al. (1986) for plant pathogenic fungi.

**Nitrate reductase analysis.** Strains with mutations at each crn locus were examined for nitrate reductase activity relative to their wild-type parent. The procedure was modified from Garrett & Cove (1976). Mutant strains and their wild-type progenitors were grown for 5–8 d on complete medium slants (8 ml in a 16 × 150 mm culture tube). Conidia from a single tube were suspended in 4 ml 2.5% (v/v) Tween 60 solution, then added to 100 ml Fusarium complete broth in a 500 ml Erlenmeyer flask. Fusarium complete broth contained 30 g sucrose, 2.5 g KN03, 1 g KH2PO4, 0.5 g MgSO4.7H2O, 10 g FeSO4.7H2O, 10 mg FeSO4.7H2O and 0.5 ml trace elements (Correll et al., 1987), 10 ml vitamin solution (Correll et al., 1987) and 1 g yeast extract in 1 litre distilled water. Cultures were grown for 2 d with rotary shaking (150 r.p.m.) at 25 °C. The resulting mycelial suspension was vacuum-filtered using a sterile milk filter disk (Kendall Agricultural Products, Boston, MA 02101, USA) in a Buchner funnel, and rinsed with 200 ml distilled water, and frozen in liquid nitrogen. Frozen samples were stored up to 1 week at −80 °C.

Crude protein was extracted from mycelium by grinding 0.5–1.5 g frozen mycelium for 5 min with 5 ml preparation buffer (Garrett & Cove, 1976) and 0.5 g silica in a frozen mortar on ice. The resulting slurry was spun for 5 min in a refrigerated microfuge and stored on ice. The clear supernatant contained soluble proteins and was the source of nitrate reductase.

Nitrate reductase activity in each crude extract was determined by measuring the production of nitrite over time. The reaction mixture contained 25 mM-Na2SO3, 50 mM-potassium phosphate (pH 7.75), 50 mM-NaNO3, 0.1 mM-flavin adenine dinucleotide and 20 pl crude extract in a total volume of 500 ml. The mixture was incubated for 10 min at 25 °C to exhaust intrinsic NADPH. The reaction was started by adding 0.4 mM-NADPH (tetrasodium salt type I), then incubated for 0, 5, 10 or 20 min at 25 °C. The reaction was stopped with 0.1 ml 25% (v/v) barium acetate, and the mixture was clarified by spinning for 1 min in a microfuge. The supernatant (0.5 ml) was mixed with 0.5 ml 1% (w/v) sulphanilamide in 20% (v/v) HCl, 0.5 ml 0.1% (w/v) N-(1-naphthy1)ethylenediamine dihydrochloride, and 1.5 ml distilled water. Nitrite levels were measured spectrophotometrically at 540 nm. Total protein in the original crude extract was determined using a diagnostic kit for colorimetric determination of total protein in serum (Sigma Diagnostic Kit no. 540). Specific nitrate reductase activity was defined as nmol nitrite produced min−1 (mg total protein)−1.

**RESULTS**

**Characterization of sectors.**

CRUN sectors could be heterokaryotic or homokaryotic (Table 1). All CRUN sectors from strains 102 and 171 were characterized, and approximately half the CRUN sectors from each strain were homokaryotic. The homokaryotic sectors (15 out of 40) contained only crn mutants,
Table 1. Composition of CRUN sectors produced spontaneously on chlorate medium by strains of F. moniliforme

<table>
<thead>
<tr>
<th>Parent strain</th>
<th>crn</th>
<th>wt</th>
<th>nit</th>
<th>wt</th>
<th>crnM</th>
</tr>
</thead>
<tbody>
<tr>
<td>102</td>
<td>7</td>
<td>1</td>
<td>7</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>124</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>133</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>171</td>
<td>7</td>
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<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>409</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>410</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* All CRUN sectors from strains 102 and 171 were examined, and two or three CRUN sectors from other strains were examined.
† crn, chlorate-resistant, nitrate-utilizing mutant; nit, chlorate-resistant, nitrate-nonutilizing mutant; wt, wild-type; crnM + nitX, molybdenum-cofactor-deficient mutant plus another nit mutant.

and single-conidium subcultures produced only CRUN colonies. We did not determine if any of the sectors that contained only crn mutants were heterokaryons composed of different crn mutants. All heterokaryotic sectors (25 out of 40) contained either a crn mutant or a nit mutant. Some sectors contained both crn and nit mutants, while other sectors also contained wild-type nuclei. Several of these crn mutants were purified a second time by culturing a single microconidium. All of these single-spore cultures retained a CRUN phenotype; no further phenotypic segregation was observed. Thus, crn mutants appear to be stable in culture. Stability was also observed during meiosis. When a crn3 mutant was crossed with a wild-type isolate, 38 out of 67 progeny were CRUN when crn3 was the male parent, and 23 of 55 progeny were CRUN when crn3 was the female parent. The 1:1 segregation ratios in the reciprocal crosses indicate that crn3 is a stable mutant with nuclear inheritance.

The phenotypes of nit mutants isolated from CRUN sectors were determined by growing the mutants on different nitrogen sources. All known nit mutant phenotypes (Nitl, Nit3, NitM) were recovered. Mutants in the NitM class were not identified to locus (nit2, nit4, nit5, nit6, nit7). No particular nit mutant phenotype was associated with the CRUN sectors.

**Linkage relationships**

crn mutants were examined for linkage to each other and to seven loci (Klittich & Leslie, 1988) affecting nitrate reduction (Tables 2 and 3). Two mutants were considered unlinked if the progeny segregated in a 1:3 ratio of wild-type recombinants:chlorate-resistant progeny. The goodness-of-fit was evaluated using a chi-square test with one degree of freedom at P = 0.05. crn1 was tightly linked to nit3; no wild-type recombinants were observed among 118 progeny. Close linkage was also found between crn2 and nit5 and between crn5 and nitl (Table 3). Loose linkage is possible between crn4 and nit6. All other loci were unlinked.

All progenies of nit mutants by crn mutants which fitted a 1:3 ratio were also consistent with a 1:2:1 ratio of wild-type : nit : crn. An exception was the nitl × crn3 cross; the progeny segregation was consistent with a 1:3 ratio but not with a 1:2:1 wild-type : nit : crn ratio due to an excess of crn progeny (15 : 20 : 24). Because the nit mutant class was generally twice the size of the crn class or the wild-type class, we conclude that the nit crn double mutants have a nit mutant phenotype.

**Nitrate reductase activity**

Strains with mutations at each of the five crn loci were compared with their wild-type progenitors for nitrate reductase activity (Fig. 1). The two crn1 mutants (derived from strains
Fig. 1. Nitrate reductase activity of crn mutants relative to their wild-type (wt) progenitors. Each bar represents the mean of three independent assays of a single mutant. Error bars represent one SD.

Table 2. Intercrosses of crn mutants

<table>
<thead>
<tr>
<th>crn1 or nit3</th>
<th>crn2</th>
<th>crn3</th>
<th>crn4</th>
</tr>
</thead>
<tbody>
<tr>
<td>crn2</td>
<td>17/46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>crn3</td>
<td>29/101</td>
<td>19/50</td>
<td></td>
</tr>
<tr>
<td>crn4</td>
<td>8/48</td>
<td>7/39</td>
<td>12/56</td>
</tr>
<tr>
<td>crn5</td>
<td>12/41</td>
<td>17/72</td>
<td>31/49</td>
</tr>
</tbody>
</table>

* All segregation ratios were consistent with 1:3 wild-type:crn using chi-square analysis, \( P \geq 0.05 \). An exception is crn3 x crn5, \( P < 0.01 \).

Table 3. Crosses of crn mutants with nit mutants

<table>
<thead>
<tr>
<th>nit mutant*</th>
<th>crn2</th>
<th>crn3</th>
<th>crn4</th>
<th>crn5</th>
</tr>
</thead>
<tbody>
<tr>
<td>nit1</td>
<td>8/46</td>
<td>15/59</td>
<td>18/54</td>
<td>1/205</td>
</tr>
<tr>
<td>nit2</td>
<td>14/40</td>
<td>9/41</td>
<td>9/52</td>
<td>12/58</td>
</tr>
<tr>
<td>nit4</td>
<td>12/51</td>
<td>17/53</td>
<td>9/43</td>
<td>19/59</td>
</tr>
<tr>
<td>nit5</td>
<td>1/103</td>
<td>14/36</td>
<td>14/41</td>
<td>16/58</td>
</tr>
<tr>
<td>nit6</td>
<td>13/58</td>
<td>12/60</td>
<td>5/48</td>
<td>11/57</td>
</tr>
<tr>
<td>nit7</td>
<td>10/43</td>
<td>14/37</td>
<td>17/54</td>
<td>21/59</td>
</tr>
</tbody>
</table>

* nit1, structural gene for nitrate reductase; nit2, nit4, nit5, nit6, nit7, genes controlling production of a molybdenum-containing cofactor (Klittich & Leslie, 1988).

† Except as noted, all segregation ratios were consistent with 1:3 wild type:chlorate-resistant using chi-square analysis \( (P \geq 0.05) \). Exceptions are nit5 x crn2 \( (P < 0.01) \), nit1 x crn5 \( (P < 0.01) \), and nit6 x crn4 \( (0.05 > P > 0.01) \).

124 and 410), the crn3 mutant (derived from strain 409) and the crn5 mutant (derived from strain 102) all had low nitrate reductase activity compared with their wild-type progenitors. The crn2 mutant (derived from strain 133) had nitrate reductase activity equal to or greater than its wild-type progenitor. The nitrate reductase activity of the crn4 mutant (derived from strain 102) was
Nine *crn* mutants each from strains 102 and 171 were tested for nitrate reductase activity and for allelism with identified *crn* loci (Table 4). *crn* mutants were considered allelic if no wild-type ascospores were obtained from the cross. Mutants at *crn1*, *crn2* and *crn3* were identified from strain 171, and mutants at all five *cm* loci were identified from strain 102. Nitrate reductase activity was assayed once for each of the *crn* mutants (Table 4) to obtain a general range of activity at each locus. *crn1* and *crn3* mutants had consistently low levels of nitrate reductase activity, and *crn2* mutants had consistently high levels of nitrate reductase activity. Ranges of activity could not be obtained for *cm4* and *crn5* because we have isolated only a single individual with each mutation. The specific activity for the wild-type strains in these assays averaged 42 nmol nitrite min\(^{-1}\) (mg protein\(^{-1}\)) for strain 171 (SD = 13.8) and 33 nmol nitrite min\(^{-1}\) (mg protein\(^{-1}\)) for strain 102 (SD = 14.3).

**DISCUSSION**

Mutants of *F. moniliforme* which utilize nitrate but are resistant to chlorate are produced spontaneously when *F. moniliforme* is cultured on medium containing chlorate. These *crn* mutants are stable through meiosis and are nuclearly inherited. The mechanism of chlorate resistance for mutants which cannot utilize nitrate (*nit* mutants) is presumably avoidance of chlorate toxicity. Chlorate is an analogue of nitrate which can be reduced to toxic chlorite by nitrate reductase, the same enzyme that catalyses the reduction of nitrate to nitrite. Cove (1976a) proposed an alternative mechanism, suggesting that chlorate mimics nitrate in mediating a shutdown of nitrogen metabolism, resulting in nitrogen starvation. *crn* mutants which have decreased nitrate reductase activity may reduce enough nitrate to permit its use as a nitrogen source, but have not sufficient activity to poison the cell through the accumulation of chlorite. The mechanism of chlorate resistance in nitrate-utilizing (*crn*) mutants has been
examined in Aspergillus nidulans. In crnA mutants of A. nidulans, conidia and young mycelia have normal levels of nitrate reductase activity but decreased nitrate uptake, conferring chlorate resistance without affecting nitrate reduction. The determination of the mechanisms of chlorate resistance in crn mutants of F. moniliforme requires further biochemical analysis.

crn mutants in F. moniliforme mapped to five unlinked loci: crn1, crn2, crn3, crn4 and crn5. The crn1 locus is closely linked to, and may be allelic with, the nit3 locus. nit3 is presumably a regulatory locus controlling induction of the nitrate reductase pathway (Klittich & Leslie, 1988). Mutants of nit3 are chlorate resistant, unable to utilize nitrate or nitrite, and do not secrete nitrite (Klittich & Leslie, 1988). Thus, they are identical in phenotype to many mutants of the regulatory loci nirA, NirL and nit-4 controlling pathway-specific nitrate reduction in Aspergillus nidulans, Septoria nodorum and Neurospora crassa, respectively (Garrett & Amy, 1978; Newton & Caten, 1988; Marzluf, 1981). crn1 mutants have low levels of nitrate reductase activity compared with their wild-type progenitors and might be leaky alleles at the nit3 locus.

The crn2 locus is closely linked to the nit5 locus. nit5 is one of five loci controlling production of a molybdenum-containing cofactor in F. moniliforme (Klittich & Leslie, 1988); mutants at nit5 have undetectable nitrate reductase activity. Mutants at crn2 differ from the other crn mutants in their level of nitrate reductase activity. In some cases, crn2 mutants have higher levels of activity than their wild-type progenitors. It is unlikely that crn2 is allelic with nit5 because inactivating nit5 should decrease rather than enhance nitrate reduction. crn2 mutants may be analogous to mutants of crnA in Aspergillus nidulans, which also have high levels of nitrate reductase activity and which are also linked to other nitrate reductase loci (Brownlee & Arst, 1983). The crnA locus of A. nidulans controls nitrate uptake in young mycelia and conidia; mutations at crnA decrease nitrate (and presumably chlorate) uptake. The crn2 locus may have a similar function in F. moniliforme.

The crn5 locus is closely linked to the nitl locus. nitl is presumably the structural locus for the nitrate reductase enzyme (Klittich & Leslie, 1988). It is possible that the single wild-type recombinant among the 200 progeny of the nitl × crn5 cross is the product of intragenic recombination or gene conversion. If so, the crn5 mutant may be a leaky mutant allele of nitl, allowing enough nitrate reduction for survival on nitrate medium but not so much that the cell is poisoned by chlorate. The low level of nitrate reductase activity in the crn5 mutant supports this hypothesis.

The crn3 and crn4 loci are unlinked to any crn or nit mutants. A crn3 mutant had a low level of nitrate reductase activity, and a crn4 mutant had a moderate level of nitrate reductase activity. Because crn3 and crn4 are not linked to any nitrate reductase loci, it is difficult to speculate about their function. The mutants could be leaky alleles of previously unidentified nit loci, such as a regulatory locus analogous to areA in Aspergillus nidulans or nit-2 in Neurospora crassa (Marzluf, 1981). A nitrate-nonutilizing mutant which may be analogous to these mutants has been identified in the related fungus Gibberella zeae (Leslie, 1987).

Chlorate-resistant, nitrate-utilizing mutants have been isolated from several other eukaryotic organisms (Dunn-Coleman et al., 1984). In plants, chlorate-resistant, nitrate-utilizing mutants are more commonly isolated than nitrate-nonutilizing mutants when chlorate resistance is the selection criterion (Braaksma & Feenstra, 1982; Marton et al., 1982). For example, over 80% of the chlorate-resistant mutants isolated from Arabidopsis thaliana were alleles of the chl-1 locus. These mutants have unaltered or enhanced nitrate reductase activity, but a decreased uptake of chlorate (Doddema et al., 1978; Braaksma & Feenstra, 1982). chl-1 mutants may be analogous to crnA of Aspergillus nidulans or crn2 of F. moniliforme. Some chlorate-resistant mutants of Arabidopsis have decreased nitrate reductase activity (Braaksma & Feenstra, 1982); these may be analogous to crn1, crn3, crn4 or crn5 of F. moniliforme. Thus, the chlorate-resistant, nitrate-utilizing mutants that we have described in F. moniliforme are not unique to Fusarium or to fungi. The ease with which these mutants can be induced and studied in F. moniliforme suggests that they may provide new insights into the metabolism of nitrate in eukaryotes.
REFERENCES


