Isolation and Genetic Analysis of Chlamydomonas reinhardtii Strains Resistant to Cadmium

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In Chlamydomonas reinhardtii, cadmium induces reduction of growth, reduction of chlorophyll content, and lethality. The toxicity was higher in a cell wall-deficient strain than in the wild type. By growing the cells on agar medium containing cadmium at concentrations inducing high lethality, stable resistant clones were isolated. The resistance was due to a nuclear mutation (cadA<sup>+</sup>) which probably preexisted in the wild-type cell population, as suggested by the fluctuation test. A double mutant (cadA<sup>+</sup> cadB<sup>-</sup>) was selected on media containing higher concentrations of cadmium. The cadB<sup>-</sup> mutation, which is unlinked to cadA<sup>+</sup>, determines a resistance intermediate between the CadA<sup>+</sup> mutant and the wild-type strain. Both cadA<sup>+</sup> and cadB<sup>-</sup> mutations are partially dominant.

Materials and Methods

Strains. All strains were derived from the 137c wild-type (WT) mating-type plus (mt<sup>+</sup>) or minus (mt<sup>-</sup>) strains of C. reinhardtii. arg<sup>-7</sup> and arg<sup>-7-7</sup> are two closely linked arginine mutations (linkage group I) determining the absence of argininosuccinate lyase activity (22, 25). CW<sub>15</sub> is a cell wall-deficient mutant strain (7).

Growth conditions and media. The algae were grown aseptically under continuous light (8,000 lx) on solidified agar medium (Difco agar; 15 g/liter). The following media were used: Tris acetate phosphate medium (TAP) (12); Tris acetate phosphate medium in which the inorganic phosphates were replaced by 1 mM glycerophosphate (TagP); TagP medium supplemented with 100 mg of arginine per liter; mineral minimal medium (21) containing (M-N) and lacking 400 mg of NH₄Cl per liter; and M-N medium containing 4 g of Difco yeast extract per liter. For the cadmium-containing media, CdCl₂ was added as a sterile solution to partially cooled agar medium after the medium was autoclaved.

Sensitivity to cadmium. The sensitivity to cadmium was estimated from growth tests on TagP agar medium containing various concentrations of CdCl₂. The TagP medium was used instead of the classical TAP medium because in the presence of inorganic phosphate, an important amount of cadmium precipitates. The sensitivity was measured in two ways. In the first method, 20 μl of cell suspension (5 × 10⁶ cells per ml) was laid over TagP agar medium plus Cd, and the plates were incubated under light for 6 days. In the second method, colonies were replica plated onto the same agar medium and growth was analyzed after 2 days.

Crosses and genetic analysis. The cells of the parental strains grown on M-N-yeast extract agar medium were suspended in M-N sterile liquid medium and incubated for 4 to 6 h under light (8,000 lx) to induce gametogenesis. The gametes of opposite mating types were mixed for 1 h, and 0.2-ml samples of the mixture were plated onto 4% agar mineral medium. The plates were incubated under light for 24 h and then stored in the dark for a 5-day maturation period. The isolation of mature zygotes, induction of meiosis, and zygote germination were carried out by the method of Levine and Ebersold (18). Genetic analysis was performed by random spore analysis, generally on 60 clones.

Isolation of diploids. Crosses were made between the

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Populations tolerant to heavy metals have been described among procaryotes as well as among lower and higher eukaryotes. In bacteria, the resistance is most often genetically determined by plasmids or transposons (for a review, see reference 30). The tolerance of yeast to copper is controlled by a multicycopy nuclear gene encoding a low-molecular-weight protein (9). In the fungi Aspergillus nidulans (6) and Neurospora crassa (19), two unlinked mutations conferring resistance to cadmium have been identified. Most varieties of higher plants growing on soils contaminated with heavy metals are genetically resistant to these metals. The resistance character may be produced by the combined action of several genes (polygenic control), as in Agrostis tenuis (11), or by a single major gene, as in Mimulus guttatus (24).

Little is known about the genetics of heavy metal tolerance in algae. In Dunaliella tertiolecta (16) and Scenedesmus acutus (32), metal-resistant strains have been isolated, but resistance is gradually lost when the cells are grown in the absence of metal. This suggests that in both cases, the tolerance results from physiological adaptation. On the contrary, a durable adaptation to metal has been described for Chlorella vulgaris (10), Euglena gracilis (1), Stigeoclonium tenue (13), or Chlamydomonas reinhardtii (27). However, in each case, no genetic analysis was carried out. Obviously, research concerning the genetics of heavy metal tolerance in algae is necessary to advance our knowledge about the control of the mechanisms involved in the resistance.

This paper describes the isolation and the genetic analysis of several C. reinhardtii clones resistant to high concentrations of Cd. This organism was chosen because it constitutes a model system for genetic studies in algae and photosynthetic eukaryotes in general.

(5) of these results have been previously published as a brief note [5].

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the sensitivities of C. reinhardtii strains to Cd

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth with CdCl₂ concn (mM) of:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>WT mt⁺</td>
<td>++</td>
</tr>
<tr>
<td>WT mt⁻</td>
<td>+++</td>
</tr>
<tr>
<td>CW₁₅ mt⁺</td>
<td>+++</td>
</tr>
</tbody>
</table>

Estimated by plating 20 μl of cell suspensions (5 × 10⁶ cells per ml) on TAP agar media containing CdCl₂.

Symbols: ++, normal growth; ++++, reduced growth (20 to 50%); ++, light green cells (=30% reduction of the chlorophyll content); +, reduced growth (50 to 95%); pen绿色 cells (=80% reduction of the chlorophyll content); +/−, numerous pale green colonies; −, dead cells.

Arg-7 and Arg-7-7 complementing haploid strains. After copulation (1 h), portions of the mixture were plated onto selective agar medium (TAP) and incubated under continuous light. The diploid character of some clones was verified by determining the mean cell volume and the mating type (22).

Determination of chlorophyll amounts. The cells were extracted with 96% ethanol. The chlorophyll content was determined in the supernatant by the method of Wintermans and De Mots (34).

Fluctuation test. Cells were cultivated in TAP liquid medium under a 12-h light-12-h dark regime (synchronous culture). When the culture reached 10⁶ cells per ml, it was diluted 1,000-fold and subdivided into 48 cultures of 5 ml each. One culture of 200 ml containing the same cell density (10⁶ cells per ml) was used as a control. The cultures were subjected to further growth in the same conditions. When they reached 4 × 10⁶ cells per ml (after 6 days), 1 ml of each 5-ml culture was withdrawn and spread onto TAP agar medium containing 0.6 mM Cd. Similarly, 48 portions (each containing 4 × 10⁶ cells) were sampled from the 200-ml bulk culture and plated on the same medium.

RESULTS

Analysis of sensitivity to cadmium. The sensitivity of wild-type mt⁺ and mt⁻ strains to cadmium was first tested on TAP agar media containing various concentrations of the metal (Table 1). At concentrations of 0.2 mM or higher, cadmium reduced growth, the ability to synthesize chlorophyll pigments, and viability. For each strain, there was a specific concentration at which all the cells died. At lower concentrations, some cells grew to produce colonies on a carpet of dead cells. Table 1 also shows that the mt⁺ strain is more tolerant than the mt⁻ strain. This was confirmed by replicating colonies of both strains on the medium containing 0.45 mM Cd. This distinguished the WT mt⁺ (positive growth) from the WT mt⁻ (no growth) strain (data not shown). The wall-less CW₁₅ mutant strain is more sensitive than the two WT strains, whose cells possess a normal wall (Table 1).

Construction of isogenic strains. In order to determine whether the small but detectable difference in sensitivity between the two wild-type strains was genetically determined, we crossed WT mt⁺ and WT mt⁻, and 120 haploid progeny clones were analyzed by replica plating for their sensitivity to 0.45 mM cadmium. The clones were distributed as follows: 61 resistant (31 mt⁺ and 30 mt⁻) and 59 sensitive (28 mt⁺ and 31 mt⁻). The 2:2 segregation indicates that the sensitivity difference between the two WT strains is determined by a pair of nuclear alleles (rcd⁺ and rcd⁻) which are linked to the mt locus (linkage group VI). A WT rcd⁻ mt⁻ clone, displaying a resistance similar to that of the original WT red⁺ mt⁺, was isolated.

Isolation of cadmium-resistant strains. In order to isolate cadmium-resistant strains and measure in parallel the percentages of surviving cells, samples (0.2 ml) of WT rcd⁺ mt⁺ cell suspensions (containing from 5 × 10⁶ to 5 × 10⁸ cells per ml) were spread over TAP agar medium with Cd (0.1 to 1.0 mM). As observed in the first experiment (Table 1), cadmium concentrations of 0.4 mM or higher induced lethality. The frequency of surviving cells dramatically decreased with increasing concentrations of Cd. Ten colonies growing on 0.4 mM Cd (survival, ±10%) and fifteen colonies growing on 0.6 to 0.9 mM Cd (survival, from 2.5 × 10⁻³ to 10⁻⁶%) were isolated and grown on TAP agar medium. They were next tested for their ability to grow on media containing several concentrations of cadmium. The results (not shown) indicated that the clones isolated on Cd at a low concentration (0.4 mM) had the same sensitivity as the WT mt⁺ strain. By contrast, the clones isolated on cadmium at high concentrations were more resistant (by about twofold) than the WT strain from which they are derived. From four independent experiments, a total of 20 clones resistant to Cd (0.8 or 0.9 mM) were isolated. After successive transfers on Cd-free agar medium for up to 1 year, all the clones maintained their resistance character.

Genetic analysis. To verify that the resistance character resulted from a genetic change, the 20 mt⁺ isolates tolerant to cadmium were crossed with WT mt⁻ cells carrying the rcd⁺ allele originally present in the WT mt⁺ strain (see above). The meiotic products were replica plated onto agar medium containing 0.7 mM Cd, a concentration which was found to be suitable for discrimination between sensitive and resistant clones. In all cases, a 2:2 segregation was obtained (data not shown), indicating that for each clone, the resistance resulted from a single nuclear mutation (cadAR⁰).

In order to determine whether one locus or several loci are involved in determining the resistance, a CadA1 rcd⁻ mt⁻ clone was first isolated from the cross CadA1 rcd⁺ mt⁺ × WT rcd⁻ mt⁻. This CadA1 rcd⁻ mt⁻ strain was then crossed with the 19 other mt⁺ resistant strains (CadA2 through CadA19). In all cases, of the meiotic progeny displayed the resistant character, indicating that all cadAR mutations were allelic. In crosses of CadA rcd⁺ mt⁺ with WT rcd⁻ mt⁻, the results indicated that the cadAR mutation was linked to the rcd locus (data not shown).

The dominance of the resistance character was further analyzed. arg-7 rcd⁺ mt⁺ strains containing the cadAR mutation present in the CadA1, CadA2, CadA3, and CadA4 clones were constructed and crossed with the arg-7-7 cadAS rcd⁻ mt⁻ strain. The resistance of the resulting diploids was intermediate between those of the parental strains (Table 2), indicating that the dominance of the cadAR mutation was partial.

Fluctuation test. In order to establish whether the resistant cells were preexisting in the initial population or were induced by the cadmium present in the medium, we used the method of fluctuation analysis (23). One WT rcd⁺ mt⁺ clone was cultivated in TAP liquid medium and then subdivided into 48 cultures as described in Materials and Methods. The numbers of colonies obtained in the different plates after 10 days are given in Table 3.

In the control (48 samples from the same 200-ml culture), the numbers of resistant clones per plate followed, as expected, a Poisson distribution; the variance did not differ significantly from the mean. On the contrary, in the 48 different small cultures, a high variance of the distribution
significantly different from the mean was observed. This non-Poisson distribution cannot be explained by physiological adaptation or mutation due to the selection agent. Instead, it means that spontaneous genetic changes occurred at random times during growth in the absence of cadmium.

Isolation of highly resistant clones. Cells of the CadAl mt⁺ strain were plated at high density (up to 2 × 10⁵ cells per plate) onto TAP agar medium containing 5 mM Cd (from 0.6 to 2.25 mM). Some of the rare colonies growing on 1.25 mM Cd were isolated, and after subsequent growth on TAP, one of the isolates, was plated on agar medium containing higher levels of cadmium. In this second step, four colonies resistant to 2 mM Cd were isolated. The clones isolated from the 1.25 and 2 mM Cd plates were next tested for their resistance levels and stabilities. The clones isolated on 1.25 mM Cd were a little more resistant than the CadAl mt⁺ strain (data not shown), but the character was lost after about 10 transfers onto Cd-free agar medium. By contrast, the clones isolated on 2 mM Cd had a resistance level about two times higher than that of the CadAl mt⁺ strain and retained their resistance after cultivation without selection pressure.

Genetic analysis of the highly resistant clones. The four clones isolated on 2 mM Cd were crossed with the WT rcd⁺ mt⁻ strain, and the haploid meiotic products were analyzed on agar medium plus Cd. In each cross, according to the Cd concentration present in the medium, the segregations of the meiotic products varied as follows: 1S(sensitive):3R(resistant) on 0.5 mM Cd; 2S:2R on 0.7 mM Cd; 3S:1R on 1.5 mM Cd; and 4S:0R on 2.2 mM Cd (data not shown). These results may be interpreted by assuming that each highly resistant clone possesses a second new nuclear mutation (cadBR) unlinked to cadA. Following this hypothesis, in the cross cadA⁺ cadBR × cadA⁺ cadB⁺, four equal classes of haploid spores are expected: cadA⁺ cadB⁺ (WT, no growth on 0.5 mM Cd), cadA⁻ cadB⁺ (no growth on 0.7 mM Cd), cadA⁺ cadB⁻ (no growth on 1.5 Cd), and cadA⁻ cadB⁻ (growth on 1.5 mM Cd and no growth on 2.2 mM Cd). The hypothesis that the cadBR mutation determines a resistance level intermediate between the WT and CadA strains was confirmed when four clones presumed to be cadA⁺ cadBR were analyzed for their Cd sensitivities (see strain cadBR in Table 2) and next crossed with a cadA⁺ cadB⁺ clone of the opposite mating type. The results indicated that, for each cross, a quarter of the haploid progeny possessed the same resistance level as the highly resistant clones isolated on 2 mM Cd.

Finally, from crosses between the CadB1 clone with the CadB2, -3, and -4 clones, it was concluded that the four cadBR mutations were allelic.

The dominance of the cadBR allele was also analyzed as described above. As for the cadA⁺ mutation, the resistance character was partially dominant (data not shown).

Expression of the resistance characters in a cell wall-deficient strain. It is well known that the cell wall plays a protective role against the metallic ions and so contributes to the metal tolerance of the organism. In the case of C. reinhardtii, we have shown that the CW₁₅ cell wall-deficient strain (CW⁻) was more sensitive to cadmium than the wild-type strains (Table 1). We wondered whether the cadA⁺ and cadB⁺ mutations can be expressed in a CW⁻ strain. If so, that means that the mutations are not involved in a modification of the wall, conferring an increased resistance to cadmium. The CW₁₅ mt⁺ strain was thus crossed with the CadA₁ mt⁻ and the CadB₁ mt⁻ strains. In each cross, among the CW⁻ haploid segregants, two approximately equal classes were observed: sensitive clones, which died on 0.4 mM Cd (as did the CW⁻ parent), and resistant clones, growing in concentrations of up to 0.5 and 0.6 mM (from crosses with CadB and CadA, respectively). This indicates that (i) cadA⁺ and cadB⁺ mutations are expressed in wallless cells and (ii) CW₁₅, cadA⁺, and cadB⁺ segregate independently. The different sensitivities of the CW₁₅ strain and its cadA⁺ and cadB⁺ derivatives were confirmed by plating cell suspensions on different Cd agar media (Table 2).

DISCUSSION

The sensitivities of C. reinhardtii strains to cadmium were tested on an agar medium (TAGP) in which the amount of insoluble cadmium was reduced. It is well known that the metal toxicity depends on the concentration of the free ions, and thus on physicochemical factors (pH and compounds able to complex or precipitate the metal) which influence this concentration (28). It is therefore difficult to compare our results with those obtained for Chlamydomonas strains (4, 15, 27) or other algae (29) with other media.

In our experimental conditions, the first signs of intoxica-

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**TABLE 2. Sensitivities of C. reinhardtii strains to Cd**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth with CdCl₂ (mM) concn cf:</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>WT</td>
<td>++</td>
</tr>
<tr>
<td>cadA⁺</td>
<td>++</td>
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<td>cadB⁺</td>
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<tr>
<td>CW₁₅</td>
<td>++</td>
</tr>
<tr>
<td>CW₁₅cadA⁺</td>
<td>++</td>
</tr>
<tr>
<td>CW₁₅cadB⁻</td>
<td>++</td>
</tr>
</tbody>
</table>

* Determined as described in Table 1, footnote a.

**TABLE 3. Distribution of resistant colonies obtained from the bulk 200-ml culture and the 48 individual 5-ml cultures after plating onto 48 plates of TAGP agar medium containing 0.6 mM CdCl₂**

<table>
<thead>
<tr>
<th>Colony source</th>
<th>No. of plates with the following no. of resistant colonies per plate:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>200-ml bulk culture</td>
<td>3</td>
</tr>
<tr>
<td>48 individual 5-ml cultures</td>
<td>10</td>
</tr>
</tbody>
</table>

* Mean number of resistant colonies per plate, 2.62; variance (x²), 2.88.

* Mean number of resistant colonies per plate, 17.10; variance (x²), 7.091.
tion of the WT cells appeared at 0.2 mM Cd. Depending on the metal concentration, three distinct effects were observed: reduction of growth rate, reduction of chlorophyll content, and lethality. In photosynthetic organisms, cadmium interferes with several metabolic activities and cell compartments, such as oxidative phosphorylation (26), mitochondrial structures (8), CO₂ fixation (2), photosystem II electron transport (20), and chlorophyll synthesis (15).

By using various concentrations of Cd, minor differences in the metal tolerance were detected between the WT mt⁺ and WT mt⁻ strains. This differential sensitivity is due to a nuclear gene present in two allelic forms (rcaD™ and rcaD⁺) and unrelated to the mating-type locus (linkage group V1). By growing the cells on high levels of metal, Cd-tolerant strains could be selected in one, two, or three steps. In contrast to the clones isolated in the second step, the tolerance so acquired was conserved for the clones isolated in the first and third steps, even after prolonged growth (up to 200 generations) in the absence of metal. This indicates that resistance results not from a physiological adaptation but rather from a genetic change.

The fluctuation test revealed that the Cd-resistant cells were not induced by the metal but preexisted in the initial population. A repeat of this experiment on a large scale would allow us to confirm this conclusion. Two major unlinked nuclear mutations (cadA™ and cadB™) conferring resistance to cadmium have been identified. The cadB™ mutants were not isolated through the first step, probably because the Cd concentration used (0.6 to 0.9 mM) for the selection was too high and thus lethal for the mutants (Table 2). Both cadA™ and cadB™ mutations are expressed in a cell-wall-deficient strain, which indicates that they do not involve a modification of the structure or composition of the wall. A CW₁₄ cadmium-tolerant strain has also been recently isolated in a Chlamydomonas strain after a prolonged growth (several months) in the presence of metal (27). In contrast to our mutants (data not shown), these cells were characterized by a low chlorophyll content. As no genetic analysis of the strain was performed, it is impossible to conclude that this phenotypical modification is resulting from a unique mutation or several independent genetic changes.

Our results also demonstrate that the cell wall plays a major role in the resistance to cadmium (Table 2), thus confirming the data of Tarmohamed and Stokes (33). This is not surprising, since the wall of C. reinhardtii possesses a high affinity for metallic cations, including cadmium (35). Since the cadA™ and cadB™ mutations are expressed in cell-wall-less cells, what are the modifications conferring the tolerance to cadmium? Various mechanisms can be hypothesized, such as (i) decreased uptake, as described for metal-resistant cells of E. gracilis (1) and Chlorella sp. (10); (ii) overproduction of metal-binding proteins, which are present notably in C. reinhardtii (5) and C. pyrenoidosa (14); (iii) increased extracellular release of organic material which can chelate free metals in solution (3); or (iv) sequestration of metal in intracellular insoluble complexes (17, 31). All these mechanisms are compatible with the partial dominance observed for both cadA™ and cadB™ mutations.

We are currently investigating the resistant strains in order to characterize the mutations at the biochemical level.

ACKNOWLEDGMENTS

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LITERATURE CITED

CADMIUM-RESISTANT C. REINHARDTI STRAINS


