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Toxic effect and adaptation in *Scenedesmus intermedius* to anthropogenic chloramphenicol contamination: genetic versus physiological mechanisms to rapid acquisition of xenobiotic resistance

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Abstract Anthropogenic water pollution is producing a challenge to the survival of phytoplankton populations. From an ecological point of view, the tolerance of these microorganisms to water pollution is of paramount importance since they are the principal primary producers of aquatic ecosystems. The adaptation of a common chlorophyta species (*Scenedesmus intermedius*) exposed to selected dose-response chloramphenicol (CAP) concentrations has been analyzed. A fluctuation analysis demonstrated that CAP-resistant cells arise due to spontaneous mutation which occurs randomly prior to the antibiotic exposure. CAP-inhibited growth and photosynthetic performance of algal cells at 0.28 mg/l, and the $IC_{50(72)}$ value was established in 0.10 mg/l for both parameters. The mutation rate from CAP sensitivity to resistance was 1.01×10^{-5} mutations per cell division, while the frequency of CAP-resistant allele in non-polluted environment was estimated to be 5.5 CAP-resistant mutants per 10^3 sensitive-cells. These results demonstrate that resistant mutants exhibit a diminished fitness until 5 mg/l of CAP, thus enabling the survival of microalgae population.

Keywords Chloramphenicol · *Scenedesmus intermedius* · Toxicity · Mutation rate · Fluctuation analysis

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Introduction

Human activities are changing biosphere-level processes (global change) and causing biodiversity crisis (Woodruff 2001; Myers and Knoll 2001). New substances are polluting water and causing environmental catastrophes in inland water systems. This is a problem of the utmost importance with basic research urgently needed to provide useful information to make future predictions, so that strategies may be designed to mitigate this environmental crisis (Ehrlich 2001). To this end, studies focused on discovering if essential microbes succumb to anthropogenic toxins are very significant (Woodruff 2001) since human activities are the greatest evolutionary force (Palumbi 2001). As microalgae are the primary producers of aquatic ecosystems (Kirk 1994; Falkowski and Raven 1997), the tolerance of these microorganisms to contaminated environments is very important from an ecological point of view.

Antibiotics play a major role in modern agriculture and aquaculture activities with their use increasing in many developed nations (Sarmah et al. 2006). These drugs are mainly administered through medicated feed. This practice may result in the antibiotics entering the environment by leaching from uneaten feeds, unabsorbed particles present in manure, or from the discharge of aquatic animals (Robinson et al. 2007). Studies performed in intensive fish farms have demonstrated that about 70–80% of applied antibiotics administered to fish as food additives end up in the aquatic environment. This may result in adverse ecological effects, including the development of resistant bacterial populations, direct toxicity to microbiota, and/or possible risks in the transfer of these antibiotic resistances to human pathogenic microbes (Rigos et al. 2004). Therefore, the overuse and misuse of certain antibiotics has

led to several adverse environmental effects and raised public concern (Sarmah et al. 2006).

Chloramphenicol (CAP) is a broad-spectrum antibiotic, commonly used in veterinary and aquaculture practice as a chemotherapeutic agent to control diseases (Uriarte et al. 2001). It prevents the synthesis of proteins by binding onto ribosomes in the same location where mRNA binds to ribosomes. Through this process, CAP also halts the synthesis of proteins in chloroplasts (Pogo and Pogo 1965; Anderson and Smillie 1966).

The main goals of this work were determine the toxic effect of CAP on microalgae, to establish the capacity of microalgae to survive in CAP contaminated environments as the result of rapid adaptation, and to verify if rapid adaptation to high doses of CAP is due to a process of physiological acclimation or genetic adaptation. CAP-resistance is a very complex problem, with important environmental and health implications (Levy 2002).

Within limits, organisms may survive in chemically contaminated environments as a result of two different processes: physiological acclimation which usually results from modifications of gene expression, and genetic adaptation by natural selection due to the occurrence of mutations which provide the appropriate resistance (Belfiore and Anderson 2001). Usually, the work of ecotoxicologists is focused toward physiological level. In contrast, rapid genetic adaptation to contaminated environments has been scarcely studied.

The statistical analysis was performed using a Luria-Delbrück Fluctuation Analysis (1943) modified as previously described (López-Rodas et al. 2001; Costas et al. 2001) which allows for differences between physiological acclimation and genetic adaptation. In previous works focused on understanding the mechanisms of microalgae adaptation to extreme natural environments (Lopez-Rodas et al. 2008a, b; Costas et al. 2007, 2008; Flores-Moya et al. 2005), fluctuation analysis proved to be useful to differentiate between these two mechanisms.

Materials and methods

Experimental organisms and culture conditions

The experiments where performed with chloroficeae *Scenedesmus intermedius* isolated from a pristine lagoon in Doñana National Park (SW Spain). This is a stock strain stored in the algal culture collection of the Complutense University (Madrid, Spain). *S. intermedius* was grown axenically in culture flasks (Greiner, Bio-One Inc., Longwood, NJ, USA) with 20 ml of BG-11 medium (Sigma Aldrich Chemie, Taufkirchen, Germany), at 20°C under continuous light of 60 μmol m^{-2} s^{-1} over a waveband of 400–700 nm. Cultures were maintained at mid-log exponential growth by serial transfers of cell inoculums to fresh medium. Prior to beginning the experiments, the cultures were re-cloned by isolating a single cell to avoid including any previous spontaneous mutants accumulated in the cultures.

Effect of CAP on the growth rate and photosynthetic performance

Chloramphenicol (CAP, ≥ 98% purity from Sigma-Aldrich Chemical Co. St. Louis, MO, USA) was used dissolved in distilled water. The toxic effect of CAP on the growth rate of *S. intermedius* was initially tested using 13 ml polystyrene sterile tubes (Sarstedt Co., Nümbrecht, Germany). No adherence to the tube walls of either chemicals or microalgae was previously checked. Neither chemicals nor microalgae were observed to adhere to the tube walls. Serial dilutions 0.05, 0.1, 0.15, and 0.2 mg/l of CAP were prepared in BG-11 medium. Eight replicates were inoculated with 10^4 cells from mid-log exponentially growing cell cultures for each concentration.

The effect of CAP was estimated after 72 h by calculating the acclimated maximal growth rate (m) in mid-log exponentially growing cells, that derives from the equation: $N_t = N_0\, e^{mt}$, where $t = 3$ days, N_t are the cell numbers at the end of the experiment and N_0 are the cell numbers at the beginning of the experiment. Therefore, m was calculated as: $m = \log_e (N_t/N_0)/t$. Acclimated maximal growth rate (m) is the Malthusian parameter of fitness under conditions of r selection (Crow and Kimura 1970; Spiess 1989). Experiments and controls were blind trials (i.e., the person counting the test did not know the identity of the tested samples) done/repeated every 24 h using a haemocytometer in an inverted microscope (Axiovert 35, Zeiss, Oberkóchen, Germany). The number of samples in each case was determined by using the progressive mean procedure (Williams 1977) which assures a counting error below 5%.

The photosynthetic response was measured as an effective fluorescence quantum yield (Φ_{PSII}) with experiments and controls done in triplicate using a ToxY-PAM fluorimeter (Walz, Effeltrich, Germany) at 72 h. Effective quantum yields were calculated as follows: $\Phi_{PSII} = (F'_m - F_t)/F'_m$, where F'_m and F_t are the maximum and the steady-state fluorescence of light-adapted cells, respectively (Schreiber et al. 1986).

A concentration causing 50% growth inhibition in algae was evaluated according to the 'area under the curve' method prescribed by the ISO (1982). Seventy-two hour mean and 100% inhibitory concentration values ($IC_{50(72)}$ and $IC_{100(72)}$, respectively) were determined by nonlinear regression analysis with all results expressed as mean ± SD. Data were presented as an inhibition percentage of growth rate and Φ_{PSII} with regard to control Statistical analysis was

performed using the computer software package GraphPad Prism v 4.0 (Graph-Pad Software Inc, USA).

Fluctuation analysis: from CAP-sensitive to CAP-resistant cells

Scenedesmus intermedius could conceivably adapt to lethal doses of CAP through a selection process of rare spontaneous mutations occurring prior to CAP exposure. Furthermore, this could transpire due to a direct and specific response to the CAP, specifically a physiological acclimation or post-selective mutations that occur as a result of CAP. Fluctuation analysis allows distinguishing between CAP-resistant cells which originated from random spontaneous mutations occurring prior to CAP exposure, and CAP-resistant cells arising through adaptation acquired during the exposure to CAP.

A fluctuation analysis consists of experiments (set 1) and its controls (set 2). In the first set of experiments 100 tubes were inoculated with a sufficiently small number of wild-type cells to ensure that no pre-existing mutants were present (in this experiment $N_0 = 10^2$ cells per tube). In the beginning of the experiment, the cultures were grown without CAP. As the cells divide, some may randomly mutate from CAP-sensitivity to CAP-resistance which gives rise to mutant clones. At the end of the growing period cultures grew until $N_t = 1.13 \times 10^5$ cells. At this point, cultures were supplemented with fresh liquid medium containing 5 mg/l of CAP (the selective agent). Such a dose corresponds approximately to 20 times the $IC_{100(72)}$, value obtained in previous acute toxicity assays (and inhibits the growth of the wild-type CAP-sensitive algae). Only CAP-resistant mutants would be able to grow under a load of 5 mg/l of CAP. Tubes were periodically checked to detect CAP-resistant cells. At the end of 2 months of culturing using CAP, which was adequate time to ensure that resistant mutant cells could generate enough progeny to be detected, all the tubes were counted.

Chloramphenicol (CAP)-resistant cells could be explained in two ways: if resistant cells arise through a direct and specific response to CAP, each cell would have a similar probability of survival and the number of CAP-resistant cells would be similar in all tubes. If so, inter-tube variation would be consistent with the Poisson model (i.e., the variance of these replicate samples would be equal to the mean). In contrast, if CAP-resistant cells arise through rare spontaneous mutations that occur prior to CAP exposure, in some cultures mutation may occur early and many of the progeny would be resistant. In other cultures, mutation could appear during a later cell division resulting in only a few resistant progeny. In still others, no mutation may occur so that all of the progeny would be sensitive. Therefore, inter-tube variation would not be consistent with the Poisson model (i.e., the variance of these replicate samples would be higher than the mean).

In set 2 (control), 25 aliquots of 1.13×10^5 cells from the same parental population were separately transferred to culture flasks containing fresh liquid medium containing 5 mg/l CAP. The number of cells in each tube was similar since variation is due only to random sampling and variation from tube to tube would be consistent with the Poisson model. Since set 2 constitutes the experimental control for the fluctuation analysis, if a similar variance/mean ratio between set 1 and set 2 is found, resistant cells would appear as a result of a direct and specific response to CAP. This could be a physiological acclimation or due to post-selective mutations that occur as a result of CAP. In contrast, if the variance/mean ratio in set 1 is higher than the variance/mean ratio in set 2 (fluctuation), this will confirm that resistant cells appear by through a rare spontaneous mutation.

In addition, the fluctuation analysis allows estimating the mutation rate (μ) by means of the P_0 estimator which represents the proportion of cultures showing no mutant colonies after CAP exposure in the first set of experiments. The P_0 estimator (Luria and Delbrück 1943) was calculated as follows:

$$P_0 = e^{-\mu(N_t - N_0)}$$

where P_0 is the proportion of cultures showing no resistant cells, N_0 is the initial cell population size, and N_t is the final cell population size. Thus, μ was calculated as (Luria and Delbrück 1943):

$$\mu = -\mathrm{Log}P_0/(N_t - N_0).$$

Characterization of CAP-resistant mutants

Chloramphenicol (CAP)-resistant cells were randomly isolated from set 1 cultures and grown to mass populations. Fitness CAP-resistant mutants and CAP-sensitive wild-type cells were characterized under conditions of selection in culture medium without CAP, as presented in a concentration-effect study. Three replicates of CAP-resistant mutants and three controls (CAP-sensitive wild-type cells) were grown in culture medium. After 4 days, cell numbers in experimental and control groups were counted using an inverted microscope (Axiovert 35, Zeiss, Oberkóchen, Germany).

Mutation-selection equilibrium

The mutation from CAP-sensitive wild-type allele to a CAP-resistant allele is recurrent. Furthermore, CAP-resistant allele is detrimental in fitness in the absence of the antibiotic. Luria and Delbrück fluctuation analysis demonstrated that new resistant mutations arise in each

generation, but most of these strains are eliminated eventually by natural selection or by chance (Crow and Kimura 1970; Spiess 1989). There is equilibrium among resistant mutants that arise by mutation, and the ones that disappear in a population in non-selective conditions. The average number of such mutants will be determined by a balance between the mutation rate and the rate of selective elimination. This is in accordance with the equation $q = (\mu/s)^{1/2}$ (Kimura and Maruyama 1966) where q is the frequency of the CAP-resistant allele and s is the coefficient of selection calculated as $s = 1 - (m_C^r/m_C^s)$ where m_C^r is the Malthusian fitness of CAP-resistant cells and m_C^s is the Malthusian fitness of CAP-sensitive cells, both of which are measured under non-selective conditions.

Results

Effect of CAP on growth rate and photosynthetic performance

Chloramphenicol (CAP) had acute toxicity on sensitive microalgae, inhibiting both cell growth and photosynthetic performance (Table 1). The $IC_{50(72)}$ values obtained for growth inhibition were similar to those obtained for $\Phi_{(PSII)}$ quantum yield assays. No statistical differences between growth rate and $\Phi_{(PSII)}$ quantum yield assays were found when comparing linear regression analysis for both parameters (Fig. 1).

Fluctuation analysis

When an analysis of fluctuation was carried out exposing wild-type cells to an inhibitory concentration of CAP (5 mg/l), cell density was reduced in set 1 and set 2 cultures due to the effect of the antibiotic. However, after further incubation for 60 days, several of the cultures were able to re-establish growth capacity demonstrating that CAP-resistant cells do occur. A high fluctuation in the number of resistant cells per culture was observed in set 1 ranging from 0 to more than 10^7 resistant-cells per culture flask. In set 2, by contrast, all cell cultures contained CAP-resistant cells in the order of magnitude of 10^7 indicating low fluctuation (Table 2). Moreover, the variance/mean ratio in set 1 was far higher than the variance/mean ratio in set 2 ($CV_{set1}/CV_{set2} = 13.26$). From this information it can be inferred that CAP-resistant cells arose as a result of a rare spontaneous mutation that occur randomly prior to CAP exposure.

The mutation rate (μ) estimated from sensitivity to resistance for CAP in *S. intermedius* was 1.01×10^{-5} mutants per cell division (Table 2).

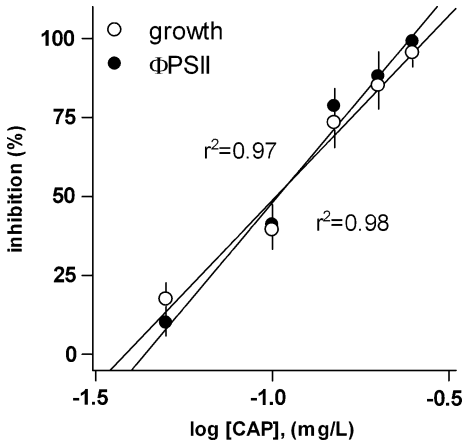

Fig. 1 Dose response relationship of chloramphenicol (CAP) to *Scenedesmus intermedius* in growth (○) and Φ_{PSII} (●) inhibition. *Points* represent means with *vertical lines* showing SD mean ($n = 8$)

Characterization of the CAP-resistant mutant

Chloramphenicol (CAP)-resistant mutants growing in BG-11 medium without CAP showed less fitness than the wild-type CAP-sensitive strains. By each doubling of the wild-type sensitive strains, CAP-resistant mutants were able only of 0.67 doublings. These relative values of fitness were used to compute the coefficient of selection (s) of

Table 1 Comparison of 72-h IC_{50} and IC_{100} values with their associated 95% confidence limits (CL), expressed in mg/l, correspondent to growth inhibition and Φ_{PSII} inhibition assays in *Scenedesmus intermedius* cells exposed to chloramphenicol (CAP)

Parameter	*n*	[CAP] (mg/l)	
		Growth inhibition	Φ_{PSII} inhibition
$IC_{50(72)}$ (CL 95%)	8	0.10 (0.09–0.12)	0.10 (0.08–0.12)
$IC_{100(72)}$ (CL 95%)	8	0.25 (0.20–0.31)	0.28 (0.21–0.38)

Table 2 Fluctuation analysis of wild-type *Scenedesmus intermedius* exposed to a growth and photosynthetic chloramphenicol-inhibitory concentration (5 mg/l)

	Set 1	Set 2
N_0 of culture replicates	100	25
N_0 (N^o cells)	100	–
N_t	3.1×10^5	
N_0 of cultures containing the following no. of chloramphenicol resistant cells		
0	4	0
10^5–10^6	3	0
10^6–10^7	16	0
10^7–10^8	77	25
CV_{set1}/CV_{set2}	13.26	
Mutation rate	1.01×10^{-5}	

CAP-resistant mutants ($s = 0.33$). By using previous μ and s values, the frequency (q) of resistant alleles as a consequence of the balance between mutation and selection was calculated. A frequency (q) of 5.5 CAP-resistant mutants per 10^3 sensitive-cells could be maintained in the absence of CAP as the result of the equilibrium between recurrent mutation and selection.

Discussion

The most commonly used antibiotics in aquaculture are: thiamphenicol, CAP, streptomycin, florfenicol, kanamycin, oxytetracycline, neomycin, and oxolinic acid. Previous studies using test systems have shown that various antibiotics remain active in wastewaters against different groups of bacteria, microflora and microfauna (Christensen et al. 2006; Quinn et al. 2008). In addition, growth inhibition effects of several antibiotics against algae and daphnids have been reported at concentrations of 5–100 mg/l (Holten Lützhoft et al. 1999; Wollenberger et al. 2000). Other authors have also recounted that several antibiotics used in intensive fish farming have various growth inhibition (EC_{50} 0.0037–3,108 mg/l) effects on algae, depending on the antibiotics and algae used (Halling-Sorensen 2000).

Results have shown that growth of *S. intermedius* is inhibited by CAP exposures. Though treatments with CAP in animal cultures are strictly prohibited, illegal utilizations are still being reported (Takino et al. 2003; Huang et al. 2006). If this antibiotic was to be used in larval culture systems (Gaunt et al. 2006; Uriarte et al. 2001), the feeding and growth of larval organisms could be adversely affected owing to the inhibition of algal populations by CAP. Furthermore, if released with effluent or overflow, CAP could potentially upset an aquatic environment. The residues of CAP from animal culture and/or human medication may result in the contamination of receiving waters.

For decades, fluctuation analysis has been considered the appropriate procedure to discriminate between resistant cells which have arisen by spontaneous mutation occurring randomly during replication of organisms prior to the exposure to a selective agent and resistant cells which arose from a direct and specific adaptation to a selective agent (i.e. CAP) (Cairns et al. 1988; Tlsty et al. 1989; Dijkmans et al. 1994). Fluctuation analysis has also been used to analyse mechanisms of algal resistance (García-Villada et al. 2002, 2004; Lopez-Rodas et al. 2007).

The large variation (fluctuation) in CAP-resistant cells in the set 1 experiment is in contrast with the minor variation in set 2 controls which unequivocally demonstrates that CAP resistance develops through a rare spontaneous mutation. This occurs prior to CAP exposure. Moreover, CAP did not stimulate the occurrence of CAP-resistant cells.

Recently, the molecular basis of CAP resistance has been revised (Siibak et al. 2009). Choramphenicol affects the assembly of both the large and the small subunits. New data suggests that CAP resistance in bacteria is due to the expression of a small resistance peptide acting on mature ribosomes (Siibak et al. 2009).

The rare mutation rate from CAP-sensitivity to CAP-resistance in *S. intermedius* (1.01×10^{-5} mutations per cell division) was found one order of magnitude higher than those previously described for the resistance to several biocides in microalgae such as formaldehyde (Lopez-Rodas et al. 2008c), erythromycin (López-Rodas et al. 2001), DCMU herbicide (Costas et al. 2001), TNT (García-Villada et al. 2002) or copper. The high mutation rate for CAP resistance may be consequence of the existence of several mechanisms of CAP resistance previously described such as reduced membrane permeability (Burns et al. 1985; Nikaido 1989), DNA ribosomal mutation (Blanc et al. 1981) and elaboration of CAP-acetyltransferase (Cohen et al. 1980). Subsequently, several mutations may confer resistance.

Chloramphenicol (CAP)-resistant cells to 5 mg/l exhibit diminished fitness in the absence of CAP compared to wild-type *S. intermedius* in the absence of CAP as the growth rate of CAP-resistant mutants is less than the wild-type CAP-sensitive cells. As a result, resistant cells are expected to be eliminated due to natural selection. However, in each generation new mutants arise creating a balance between mutation rate and selective elimination since mutation is recurrent. At the same time, there can be a certain number of mutant cells that will have not been eliminated yet. Thus, rare spontaneous mutation from CAP-sensitivity to CAP-resistance seems to be sufficient to assure the survival of microalgae populations in contaminated environments until 5 mg/l of CAP.

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