Antimicrobial cocktails to control bacterial and fungal contamination in *Chlamydomonas reinhardtii* cultures

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BioTechniques 60:145–149 (March 2016) doi 10.2144/000114392

Keywords: microalgae; contamination; bactericides; fungicides; antimicrobial cocktail

Supplementary material for this article is available at www.BioTechniques.com/article/114392.

Chlamydomonas reinhardtii is a unicellular green alga widely used for research in photosynthesis, cell cycle regulation, ciliary biogenesis, and other physiological processes. Sterile cultures are needed for these studies, but contamination from bacteria and fungi occurs frequently. Although the One-shot Solution cocktail consisting of carbendazim, ampicillin, and cefotaxime has been developed for removing these contaminants from algal cultures, it is not always effective. Here we report two new antimicrobial cocktails for treating mixed bacterial and fungal contamination of *Chlamydomonas* cultures. A combination of the bactericide nalidixic acid with one of two fungicides, azoxystrobin or tebuconazole, was more effective than the One-shot Solution cocktail. In some of our tests, we find that alternating use of our new cocktails with One-shot Solution is needed to remove obstinate contaminants.

Microalgae are widely used in scientific research and industrial applications, which include studies of photosynthesis and organelle biogenesis; the production of biodiesel fuel, foodstuffs, and medicine; and sewage treatment (1–3). *Chlamydomonas reinhardtii*, a unicellular green alga, is a model organism used in a variety of studies (4). For example, it has been widely used for studying cilia and flagella, important organelles that play a role in human health and disease. Intraflagellar transport (IFT), which is required for ciliary assembly and maintenance in

almost all ciliated organisms, including humans, was first discovered and has been best characterized in *C. reinhardtii* (5–7). These discoveries in *C. reinhardtii* have broad medical implications (8,9). In addition, *C. reinhardtii* is also used in studies of lipid metabolism for biofuel production (10,11).

Sterile culture of *C. reinhardtii* is required not only for basic research but also for applied studies. Microbial contamination may ruin the culture stock and alter experimental data, leading to misinterpretation. For applied

studies, such contamination may also affect the yield and quality of the final products. *C. reinhardtii* can grow by autotrophy, heterotrophy, or mixotrophy. Under experimental growth conditions, mixotrophic growth with a supplement of organic carbon such as acetate is usually employed; however, cells under mixotrophic growth conditions are prone to microbial contamination. Cell cultures are routinely stored on agar plates for a few weeks to several months. In addition, cells are frequently transferred from one condition to another during experiments. Cell handling and storage also frequently result in contamination from bacteria and fungi.

Once cultures are contaminated, they should be decontaminated. At present, there are a few approaches to accomplish this. The streaking method involves streaking a few cells on a new agar plate to isolate uncontaminated single clones. The centrifugal method involves repeated washing by low-speed centrifugation (600 × g). However, neither of these methods are effective for heavy contamination, especially contamination by sticky microorganisms. Previous studies found that fungicides such as carbendazim, thiophanate-methyl, and benomyl can remove fungal contaminants from *C. reinhardtii* cultures (12). As contamination is often caused by fungi as well as bacteria, Pan et al. found that the combination of carbendazim, ampicillin, and cefotaxime, termed the One-shot Solution, can effectively remove bacterial and fungal contaminants (13). However, these existing methods for removing microbial contaminants are not always effective. The source of contamination is different in different laboratory environments. Clearly, controlling contamination is complex and challenging, and additional antimicrobial strategies would be helpful.

Nalidixic acid is a common bactericide used in laboratories studying fungi. Azoxystrobin, tebuconazole, pyrimethanil, hymexazol, and chlorothalonil are fungicides widely used in agriculture; however, they have not yet

METHOD SUMMARY

Here we report two improved antibiotic/antifungal cocktails (nalidixic acid/azoxystrobin and nalidixic acid/tebuconazole) for eliminating contaminating bacteria and fungi from *Chlamydomonas reinhardtii* cell cultures. These cocktails could eliminate obstinate contaminants that are not removed by the One-shot Solution cocktail.

been used for controlling fungal contaminants in *C. reinhardtii* cultures. Nalidixic acid has broad spectrum antibacterial activity and kills the majority of Gram-negative bacteria, up to 99% of *E. coli* strains (14). Thus, it is a potentially useful antibiotic for controlling laboratory bacterial contaminants. Azoxystrobin is a member of the strobilurin group of compounds, which have broad-spectrum activity against fungi (15). Strobilurins kill fungi by inhibiting respiration and energy synthesis (16). Tebuconazole inhibits ergosterol biosynthesis, which is required for fungal membrane structure and fluidity (17). Pyrimethanil, hymexazol, and chlorothalonil are also potential antifungal agents with different mechanisms against diverse fungi (18–20). Therefore, we tested these chemicals in new antibiotic/antifungal cocktails for treatment of microbial contamination of *C. reinhardtii* cultures.

C. reinhardtii strain 21 gr (CC-1690; Chlamydomonas Resource Center, University of Minnesota, St. Paul, MN), a widely used experimental strain, was cultured on 1.5% TAP agar plates at $23 \pm 0.5^\circ\text{C}$ under 14/10 h light/dark cycles with a light intensity of 8000 lx (21). Nalidixic acid (15 mg/mL) (Sangon Biotech, Shanghai, China) was dissolved in 0.4 M NaOH. Ampicillin (100 mg/mL) and cefotaxime (50 mg/mL) (Sangon Biotech) were dissolved in water. All bactericides were filter sterilized, aliquoted and stored at -20°C as stocks solutions. The fungicides, including azoxystrobin,

Figure 1. Effects of bactericide/fungicide cocktails on the removal of microbial contaminants from *Chlamydomonas reinhardtii* cultures. Contaminated and non-contaminated cultures grown on plates were streaked on new plates with or without the indicated fungicides and bactericides. After 5–7 days, the effects of the cocktails were examined. (A) Control plate. (B) Plate with the One-shot Solution cocktail composed of Amp, Cef, and Car. (C) Plate with Azo and NC. (D) Plate with Teb and NC. 1: uncontaminated cultures; 2–4: contaminated cultures containing fungi and bacteria. Amp: ampicillin; Cef: cefotaxime; Car: carbendazim; Azo: azoxystrobin; NC: nalidixic acid; Teb: tebuconazole.

tebuconazole, pyrimethanil, hymexazol, and chlorothalonil (Bayer, Leverkusen, Germany), were suspension concentrates as originally provided by the vendor,

while carbendazim (Sigma, St. Louis, MO) was dissolved in medium at a final concentration of $40 \mu\text{g/mL}$. The bactericides were added to the agar medium after autoclaving, while the fungicides were added before autoclaving. The final concentrations are listed in Table 1; nalidixic acid, ampicillin, cefotaxime, and carbendazim were used at the recommended concentrations. Cell cultures of *C. reinhardtii* were inoculated on these agar plates containing different combinations of antimicrobials. Test contaminants were randomly collected from contaminated cultures or from the laboratory environment. Cell growth was scored visually as shown in Table 1. Photo images (captured by a Galaxy S5 camera from Samsung Electronics, Seoul, Korea) were processed with Adobe Photoshop and Illustrator software (Adobe Systems Incorporated, San José, CA).

As shown in Table 1, the cocktail of ampicillin, cefotaxime, and carbendazim did not inhibit the growth of *C. reinhardtii* and had moderate antibiotic

Table 1. Effects of bactericide/fungicide cocktails on the growth of *Chlamydomonas reinhardtii* and the removal of microbial contaminants.

Organism	Antimicrobial cocktails		Fungicide concentration ($\mu\text{g/mL}$)			
	Bactericide	Fungicide	10	20	50	100
<i>Chlamydomonas reinhardtii</i>	Nalidixic acid ¹	Azoxystrobin	+++	+++	++	+
		Tebuconazole	+++	+++	+	-
		Pyrimethanil	+++	+++	++	+
		Hymexazol	+	+	-	-
		Chlorothalonil	-	-	-	-
		Carbendazim ²	ND	ND	+++	ND
	Ampicillin ³ + Cefotaxime ⁴	Carbendazim	ND	ND	+++	ND
Contaminants	Nalidixic acid	Azoxystrobin	-	-	-	-
		Tebuconazole	-	-	-	ND
		Pyrimethanil	+	+	+	+
		Hymexazol	++	++	ND	ND
		Chlorothalonil	ND	ND	ND	ND
		Carbendazim	ND	ND	+	ND
	Ampicillin + Cefotaxime	Carbendazim	ND	ND	+	ND

+++ , growth with no obvious inhibition; ++ , growth with slight inhibition; + , growth with obvious inhibition; - , no growth; ND , not determined.

^{1,2,3,4} The concentrations of nalidixic acid, carbendazim, ampicillin, and cefotaxime were always 15 $\mu\text{g/mL}$, 40 $\mu\text{g/mL}$, 500 $\mu\text{g/mL}$, and 100 $\mu\text{g/mL}$, respectively.

and antifungal activity against contaminants. The new cocktails of nalidixic acid/azoxystrobin, nalidixic acid/tebuconazole, and nalidixic acid/pyrimethanil did not inhibit algal growth at 20 µg/mL. When the concentration was >20 µg/mL, slight inhibitory effects were observed for all 3 cocktails. The other two cocktails, nalidixic acid/hymexazol and nalidixic acid/chlorothalonil, were inhibitory for algal growth at all tested concentrations.

In addition to the evaluation of algal growth, the effects of these antimicrobials on microbial contaminants was also evaluated (Table 1). The specific types of bacteria and fungi in the contaminated cultures were not determined because contaminants vary greatly under different laboratory conditions. The nalidixic acid/azoxystrobin cocktail was effective at all tested concentrations against fungal growth. Given the inhibition of algal growth described above, the recommended concentrations for the nalidixic acid/azoxystrobin cocktail are 10 µg/mL and 20 µg/mL. The nalidixic acid/tebuconazole cocktail effectively blocked fungal growth at 10, 20, and 50 µg/mL. In light of its effect on algal growth, the recommended concentrations for the nalidixic acid/tebuconazole cocktail are 10 µg/mL and 20 µg/mL. The nalidixic acid/pyrimethanil or nalidixic acid/hymexazol cocktails were not fully effective at controlling microbial contaminants, and the efficacy of the nalidixic acid/chlorothalonil cocktail was not examined because it inhibits algal growth. These results demonstrate that the cocktails containing nalidixic acid with either azoxystrobin or tebuconazole could be used for eliminating bacterial and fungal contaminants from *C. reinhardtii* cultures. In contrast, cocktails containing ampicillin/cefotaxime/

carbendazim and nalidixic acid/carbendazim were less effective against these contaminants.

The effects of these antimicrobial cocktails are listed in Figure 1. *C. reinhardtii* cells contaminated by bacteria and/or fungi were streaked onto the indicated solid plates. After growth at $23 \pm 0.5^\circ\text{C}$ for 5–7 days, plates were examined for the effects of the different cocktails on the removal of contaminants. Mixed contamination was completely reduced on the plates that were supplemented with nalidixic acid/azoxystrobin or nalidixic acid/tebuconazole, while the ampicillin/cefotaxime/carbendazim cocktail produced only a limited reduction of mixed contaminants. In particular, the amounts of some contaminating fungi were barely reduced with the latter cocktail, while they were fully controlled by azoxystrobin or tebuconazole. These results indicate that the key for controlling microbial contamination is to suppress fungal contamination, which is more difficult to control due to fungal spores. To further confirm that these new cocktails removed contaminants, cells grown on plates supplemented with the cocktails were regrown on TAP agar plates. After 7 days, the cells had grown normally without any contamination (Supplementary Figure S1). Thus, the new cocktails could be used for the removal of microbial contaminants. The above results also verified that these new antibiotic/antifungal cocktails are more effective than the previously described One-shot Solution cocktail and that they could be alternative choices for eliminating mixed microbial contamination.

Our findings demonstrate that these new combinations of bactericides and fungicides are more economical, conve-

nient, and effective at eliminating mixed contamination by bacteria and fungi of *C. reinhardtii* cultures, with nalidixic acid used to remove bacteria and azoxystrobin or tebuconazole used to eliminate fungi. These cocktails are predicted to be effective for treating contamination in other algal cultures. For the complete control of bacterial and fungal contamination, we suggest that the antimicrobial cocktails (1. ampicillin/cefotaxime/carbendazim; 2. nalidixic acid/azoxystrobin; 3. nalidixic acid/tebuconazole) should be applied alternately to avoid antimicrobial resistance. When heavy contamination occurs, streaking cultures on agar plates two or more times, with alternate application of the antimicrobial cocktails, is required to get rid of any contamination. Finally, cells that are decontaminated should be re-streaked on a TAP agar plate without any antimicrobials before storage or use in new experiments.

Author contributions

L. W. and F. Y. designed and performed the experiments. H. C., Z. F., and Y. Z. performed the experiments. J. L. and Y. Z. provided equipment and financial support, and contributed to discussion. L. W. conceived the idea for the study and wrote the manuscript.

Acknowledgments

This research was supported by the Natural Science Foundation of China (31501087), the Natural Science Foundation of Jiangsu Province (BK20150230), the Natural Science Foundation for Colleges and Universities in Jiangsu Province (15KJB180005), the Scientific Research Support Program for Teachers with Doctor's Degrees at Jiangsu Normal University (13XLR030), and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). We thank Junmin Pan for discussion and revision of the manuscript.

Competing interests

The authors declare no competing interests.

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Received 25 September 2015; accepted 03 December 2015.

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