CHAPTER SIXTEEN

A Simple Assay for Screening Microorganisms for Chalkophore Production

Sukhwan Yoon,[*,1] Alan A. DiSpirito,[†] Stephan M. Kraemer,[‡] *and* Jeremy D. Semrau[*]

Contents

Abstract

Recently, methanotrophs were found to exude a chalkophore, that is, a metal ligand with great affinity and specificity to copper. A rapid screening method for chalkophore expression was developed by adopting the chrome azurol S (CAS) assay originally used for detecting siderophore production in diverse groups of bacteria and fungi. In this assay, iron(III) chloride was replaced with copper(II) chloride. Both liquid and agar plate versions of the Cu–CAS assay can be used to examine the activity of either isolated methanobactin or to screen organisms for production of a chalkophore. Although here we describe the use of this assay to screen methanotrophs for chalkophore production, it can be modified as necessary to screen other organisms for chalkophore production as well.

* Department of Civil and Environmental Engineering, The University of Michigan, Ann Arbor, Michigan, USA
† Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, Iowa, USA
‡ Department of Environmental Geosciences, University of Vienna, Althanstrasse, Vienna, Austria
1 Current Address: Department of Biogeochemistry, Max Planck Institute for Terrestrial Microbiology, Marburg, Germany

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Many siderophores can also bind copper in the presence of CAS. Therefore, this assay should be done in conjunction with the original iron–CAS assay to determine if any positive Cu–CAS assay results are due to nonspecific binding of copper by a siderophore. This inexpensive assay may also aid in analyses of the genetics of chalkophore synthesis.

1. Introduction

It is well-known that copper plays a significant role in methanotrophic metabolism, not only controlling the relative expression of particulate and soluble methane monooxygenases (pMMO and sMMO, respectively), but also affecting the activity of pMMO, production of intracytoplasmic membranes, and expression of other enzymes, for example, different forms of the formaldehyde dehydrogenase (Semrau, *et al.*, 2010). As such, it was long suspected that methanotrophs had a copper-specific uptake mechanism, or chalkophore, with the first example, methanobactin from *Methylococcus capsulatus* Bath, reported in 1996 as the copper-binding compound (cbc; Zahn and DiSpirito, 1996).

This unique compound, analogous to a siderophore, was found to utilize oxazalone rings for metal binding (Behling, *et al.*, 2008). Although some physicochemical properties of methanobactin, for example, affinity to copper and molecular structure, have been reported (Choi *et al.*, 2006a,b, 2008; Kim *et al.*, 2004), there has not been any significant progress in characterizing either the diversity of organisms that can express such compounds, or the genetics underpinning the synthesis of chalkophores, including methanobactin from *Methylosinus trichosporium* OB3b.

Such data are important as empirical evidence suggests that methanobactin may be involved in regulation of expression of methane monooxygenases in *M. trichosporium* OB3b (Choi *et al.*, 2010; Knapp *et al.*, 2007; Morton *et al.*, 2000). If methanotrophs produce different chalkophores with varying copper affinity, it may play a role in regulating methanotrophic community structure. Furthermore, it has been found that methanobactin from *M. trichosporium* OB3b can reduce Au(III) to Au(0), forming gold nanoparticles, and thus can have significant industrial potential (Choi *et al.*, 2006a). The reported yields of methanobactin are less than 60 mg/L of spent medium, however (Choi *et al.*, 2008), and the potential use of methanobactin in such industrial applications will require larger amounts of methanobactin. One strategy to increase the yield of methanobactin production would be the heterologous expression of genes involved in methanobactin synthesis in a less fastidious host such as *Escherichia coli*.

The lack of simple and economic methods to screen for a wide variety of microorganisms has limited our ability to determine how wide-spread the

ability to produce a chalkophore might be. For example, do all methanotrophs require such a mechanism to collect copper? Do closely related cells such as ammonia-oxidizing bacteria have a similar active copper-uptake system? Furthermore, the lack of a simple screening assay has limited our ability to deduce the genetics of methanobactin synthesis through the use of high-throughput techniques to screen putative chalkophore-minus mutants in organisms known to produce such a compound.

To address these problems, we have devised a novel method to qualitatively screen for production of chalkaphores through a simple plate assay (Yoon *et al.*, 2010). This assay was developed by adopting the chrome azurol S (CAS) assay for siderophore production (Schwyn and Neilands, 1987), to instead screen cultures for chalkophore production by substituting copper for iron. In the original assay, a blue complex is formed between iron and CAS in the presence of a detergent, hexadecyltrimethylammonium bromide (HDTMA). The removal of iron by a competing ligand, for example, a siderophore, results in a color change, typically from blue to orange. Here copper was substituted for iron, as CAS also has a high affinity for copper with a blue complex also being observed, with a similar color change observed when copper is abstracted by competing ligands.

2. Cu–CAS Assay for Chalkophore Detection

The Cu–CAS assay was developed from the popular CAS assay originally developed to assay for the presence of siderophore production (Schwyn and Neilands, 1987). As in the original CAS assay, CAS binds copper with relatively high affinity ($\log K = 13.2$; Cha and Abruna, 1990) when there is no competing ligand in the medium. As the only characterized methanobactin to date has much higher affinity towards copper than CAS, that is, $3.3 \times 10^{34} \pm 3.0 \times 10^{11}\ M^{-1}$ (Choi, *et al.*, 2006a), abstraction of copper from CAS should occur as methanobactin outcompetes CAS in binding copper. In the Cu–CAS assay described here, the colorimetric change associated with the loss of copper from CAS is used for detecting the presence of the methanobactin.

2.1. Preparation of Cu–CAS solution

Cu–CAS solution for detection of methanobactin synthesis in methanotrophs is prepared with nitrate mineral salt medium (NMS; Whittenbury *et al.*, 1970) and CAS and HDTMA (Sigma, St. Louis, MO). It should be noted that this assay can be easily modified for other organisms by substituting the appropriate growth medium as long as the color of the medium does not interfere with the color of Cu–CAS and no significant competition of

trace nutrient metal ions for complexation by CAS occurs. Below we provide step-by-step instructions for preparing Cu–CAS solutions for screening methanotrophs for chalkophore production.

1. To produce 500 mL of Cu–CAS solution with 50 μ*M* Cu^{2+}, first prepare NMS medium by adding 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KNO_3, 0.1 g $CaCl_2 \cdot 2H_2O$, 50 μL of 3.8% (w/v) Fe–EDTA, 250 μL of 0.1% (w/v) $NaMoO_4 \cdot 2H_2O$ to 450 mL of distilled deionized water (>18 MΩ·cm; Whittenbury *et al.*, 1970). Then add 0.5 mL of trace element solution (50 mg/L $FeSO_4 \cdot 7H_2O$, 40 mg/L $ZnSO_4 \cdot 7H_2O$, 2 mg/L $MnCl_2 \cdot 7H_2O$, 5 mg/L $CoCl_2 \cdot 6H_2O$, 1 mg/L $NiCl_2 \cdot 6H_2O$, 1.5 mg/L H_3BO_3, and 25 mg/L EDTA).
2. Prepare stock solutions of $CuCl_2$ (Fisher Scientific, Pittsburg, PA), CAS, and HDTMA at concentrations of 5, 1.05, and 2.625 m*M*, respectively in distilled deionized water (>18 MΩ·cm). Stir these stock solutions until all solids are dissolved and the solutions are well-mixed.
3. Add 25 mL of the CAS stock solution to 5 mL of the $CuCl_2$ stock solution. Next add 20 mL of HDTMA under stirring for final concentrations of 0.5, 0.525, and 1.050 m*M* of, Cu^{2+}, CAS, and HDTMA, respectively. This 10× stock solution of Cu–CAS should have a purple color at this stage.
4. Sterilize both the NMS growth medium and 10× Cu–CAS solution via autoclaving for 40 min.
5. Allow both the NMS growth medium and 10× Cu–CAS solution to cool to room temperature.
6. Pipette 50 mL of the 10× Cu–CAS concentrate into 450 mL of NMS growth medium.
7. Add 5 mL of sterile vitamin stock solution (20 mg/L biotin, 2.0 mg/L folic acid, 5.0 mg/L thiamin HCl, 5.0 mg/L Ca pantothenate, 0.1 mg/L vitamin B_{12}, 5.0 mg/L riboflavin, and 5.0 mg/L nicotiamide; Lidstrom, 1988) to the combined NMS growth medium and Cu–CAS solution.
8. Buffer the mixture to pH ∼ 6.8 by adding 5 mL of sterile phosphate buffer (26 g/L KH_2PO_4 and 62 g/L $Na_2HPO_4 \cdot 7H_2O$).
9. Distribute the NMS Cu–CAS mixture as 5-mL aliquots into 20 mL vials. The vials can be sealed with rubber butyl stoppers (National Scientific Co. Duluth, GA) if isolation from the atmosphere is necessary.
10. Solutions of interest, for example, concentrates of isolated methanobactin or spent microbial growth medium can be screened at this point by adding volumes less than 500 μL. A color change from bright blue to yellow should be observed shortly after addition, as well as substantial changes in the UV/Vis absorption spectra, particularly at the wavelengths associated with the heterocyclic rings, for example, 340 and 394 nm for methanobactin from *M. trichosporium* OB3b (Fig.16.1).

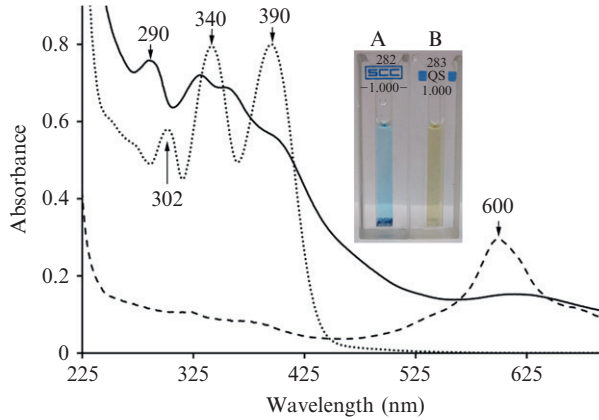

Figure 16.1 UV–visible absorption spectra of 50 μ*M* of methanobactin from *M. trichosporium* OB3b (▪ ▪ ▪ ▪ ▪), 50 μ*M* Cu–CAS (- - - - - -), and 50 μ*M* methanobactin from *M. trichosporium* OB3b plus 50 μ*M* Cu–CAS (————) after 5 min incubation at room temperature. Insert: (A) 50 μ*M* Cu–CAS; (B) 50 μ*M* methanobactin from *M. trichosporium* OB3b plus 50 μ*M* Cu–CAS.

Growth of methanotrophs in liquid NMS–Cu–CAS solution is unlikely due to the toxicity of HDTMA. Thus, this liquid assay may best be used as an initial screen to check for methanobactin production of methanotrophs grown under different growth conditions.

2.2. Preparation of Cu–CAS agar plates

As methanotrophic growth can be significantly compromised by the presence of the detergent used in the standard CAS assay, that is, HDTMA, the split-CAS assay devised by Milagres *et al.* (1999) was adapted for detecting the excretion of methanobactin from growing methanotrophs. Here split plates are made with one half containing Cu–CAS/NMS agar and the other with NMS agar only. Strains are streaked on the NMS agar, and methanobactin production is determined from color changes in the Cu–CAS/NMS agar due to diffusion of methanobactin. This assay does not inhibit methanotrophic growth and can be used to detect methanobactin production as the color changes are visible typically within 2 weeks. Below are step-by-step instructions for preparing split Cu–CAS/NMS and NMS agar plates to screen methanotrophs for chalkophore production.

1. Prepare 450 mL of NMS growth medium as described in step 1 of Section 2.1. Add 7.5 g Bacto agar (Bectron Dickinson, Franklin Lakes, NJ).
2. Prepare a 10× stock solution of Cu–CAS as described in steps 2 and 3 of Section 2.1.
3. Autoclave the NMS agar and 10× Cu–CAS solution as described in step 4 of Section 2.1.

4. Allow both the NMS agar and 10× Cu–CAS solution to cool to ~50 °C.
5. Carefully add 50 mL of the 10× Cu–CAS concentrate to 450 mL NMS agar.
6. Add vitamin and phosphate buffer solutions as described in steps 7 and 8 of Section 2.1.
7. Pour the Cu–CAS/NMS agar into standard petri dishes. After the agar has completely solidified, carefully remove half with a sterilized razor.
8. Prepare an additional 450 mL of NMS growth medium as described above in Section 2.1. Add 7.5 g Bacto agar. Autoclave the NMS agar for 40 min and allow to cool to ~50 °C.
9. Add vitamin and phosphate buffer solutions as described in steps 7 and 8 of Section 2.1.
10. Add the desired copper concentration to the NMS agar using a sterile stock solution of 10 m*M* $CuCl_2$. Previous results have shown that copper concentrations ranging between 0 and 10 μ*M* do not have any impact on the results of the experiments (Yoon *et al.*, 2010). It is recommended that at least 1 μ*M* of copper be added to the NMS agar as copper limitation may result in repressed growth of some methanotrophic strains.
11. Carefully pour the NMS agar into the empty space in the agar plate created in step 7 above. The surface of both the Cu–CAS/NMS agar and NMS agar should be level.
12. Streak methanotroph(s) of interest on the NMS agar half of the plate. Streaking methanotrophs onto the NMS agar only will prevent inhibition of microbial growth by HDTMA as there is no direct contact. It is important that cells be streaked as closely as possible to the boundary of the Cu–CAS/NMS and NMS agars, however, to reduce the time required for any chalkophore produced to diffuse into the Cu–CAS/NMS agar.
13. Incubate split Cu–CAS/NMS and NMS agar plates in a sealed container with a 1:1 air-to-methane ratio at the optimal growth temperature of the methanotroph(s) to be tested. A color change from blue to yellowish-orange should begin to appear on Cu–CAS/NMS agar within 2 weeks if a chalkophore is produced (Fig.16.2).

3. Fe–CAS Assay for Detecting Nonspecific Binding of Copper from Cu–CAS by a Siderophore

Although much more specific to iron, some siderophores also bind copper with a relatively high affinity. For example, the bacterial siderophore deferoxamine-B forms a copper-complex with a 1:1 log formation constant

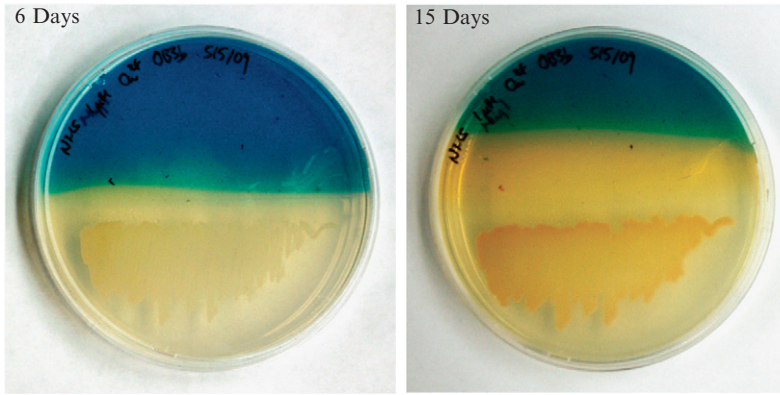

Figure 16.2 An example of the Cu–CAS plate assay performed with *M. trichosporium* OB3b. The copper concentration in the NMS medium was 1 μ*M*. Within ~15 days from inoculation, color change from blue to yellow was obvious in the Cu–CAS/NMS agar due to the production and diffusion of methanobactin from *M. trichosporium* OB3b streaked on NMS agar.

of 14.1 (Martell *et al.*, 2001) that is high enough to abstract copper from CAS (Yoon *et al.*, 2010). Therefore, separate Fe–CAS assays are required to confirm that positive results from Cu–CAS assay are not due to production of siderophores. Some methanotrophs, for example, *M. trichosporium* OB3b and *Methylomicrobium album* BG8, have been found to produce siderophores (Yoon *et al.*, 2010). Thus the standard Fe–CAS assay should be used to examine the possible production of a siderophore by methanotrophs as described below. We would like to note that the following procedures were originally developed by Schwyn and Neilands (1987) for detection of siderophore production, and are optimized here for screening of methanotrophs.

3.1. Preparation of liquid Fe–CAS

1. Prepare 450 mL of NMS solution as described in step 1 of Section 2.1. Add 15 g of PIPES and stir until completely dissolved. Adjust the pH of the NMS solution to 6.8 using 50% (w/v) NaOH. *Note*: Phosphate buffer cannot be used in Fe–CAS assays, as phosphate competes for Fe (III) bound to CAS and can cause a significant color change (Schwyn and Neilands, 1987).
2. Prepare stock solutions of CAS, $FeCl_3$ (Fisher Scientific, Pittsburg, PA), and HDTMA at concentrations of 1.05, 5, and 2.625 m*M*, respectively using distilled deionized water (>18 MΩ·cm). Stir until all solids are dissolved and the solutions are well-mixed.

3. Add 25 mL of the CAS stock solution to 5 mL of the $FeCl_3$ stock solution. Next add 20 mL of HDTMA under stirring for final concentrations of 0.5, 0.525, and 1.050 m*M* of Fe^{3+}, CAS, and HDTMA, respectively. This 10× stock solution of Fe–CAS should have a dark blue color at this stage.
4. Sterilize the NMS medium and 10× Fe–CAS via autoclaving for 40 min.
5. Pipette 50 mL of the 10× Fe–CAS concentrate into 450 mL of NMS growth medium.
6. Distribute the Fe–CAS/NMS solution as 5-mL aliquots into 20 mL vials. The vials can be sealed with rubber butyl stoppers if isolation from the atmosphere is necessary.
7. Add materials to be tested as described in step 10 of Section 2.1. If a siderophore or other iron chelator, for example, deferoxamine-B, is present in the medium, the blue tint of the Fe–CAS solution will change to yellow, while when methanobactin from *M. trichosporium* OB3b is added, a greenish color is observed (Fig.16.3). It should also be noted that collecting the UV/Vis absorption spectra can also serve as an effective methodology to determine if a siderophore is present as the peaks associated with heterocyclic rings (340 and 394 nm for *M trichosporium* OB3b) are still apparent in the presence of Fe–CAS, while they diminish in the presence of Cu–CAS (Fig.16.1).

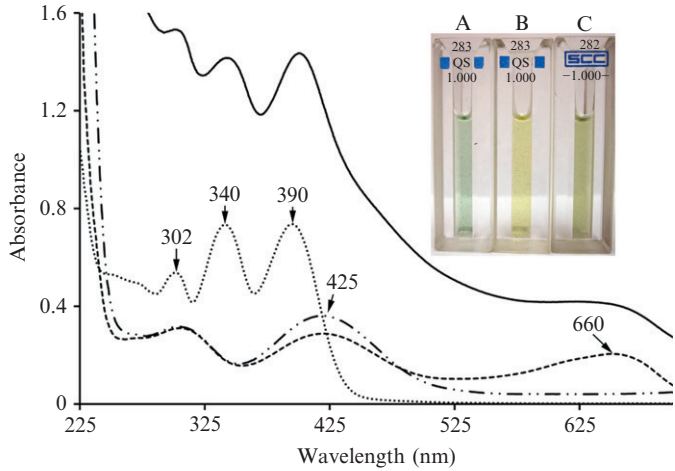

Figure 16.3 UV–visible absorption spectra of 50 μ*M* OB3b–mb (▪ ▪ ▪ ▪ ▪), 50 μ*M* Fe–CAS (- - - - -), 50 μ*M* Fe–CAS plus 50 μ*M* deferoxamine-B (— • • —), and 50 μ*M* methanobactin from *M. trichosporium* OB3b plus 50 μ*M* Fe–CAS (————) after 5 min incubation at room temperature. Insert: (A) 50 μ*M* Fe–CAS, (B) 50 μ*M* Fe–CAS plus 50 μ*M* deferoxamine-B, (C) 50 μ*M* methanobactin from *M. trichosporium* OB3b plus 50 μ*M* Fe–CAS.

3.2. Preparation of Fe–CAS agar plates

A Fe–CAS plate assay can be used for detecting siderophore production that may give false positive results for chalkophore production when using the Cu–CAS plate assay. Siderophore production can be repressed if sufficient iron is present in the growth medium (Crosa, 1989) and the standard NMS agar as used in the split Cu–CAS/NMS agar plates contains an appreciable amount of iron. Thus, it is unlikely that siderophores will interfere with the Cu–CAS assay, but such an assumption should be tested by screening for siderophore production using Fe–CAS/NMS agar plates. Below are step-by-step instructions for preparing split Fe–CAS/NMS and NMS agar plates to screen methanotrophs for siderophore production.

1. Prepare 450 mL of NMS growth medium as described in step 1 of Section 3.1. Adjust the pH of the NMS solution to 6.8 using 50% (w/v) NaOH. Add 7.5 g Bacto agar.
2. Prepare a 10× stock solution of Fe–CAS as described in steps 2 and 3 of Section 3.1.
3. Sterilize the NMS agar and 10× Fe–CAS via autoclaving for 40 min.
4. Allow both the NMS agar and 10× Fe–CAS stock solution to cool to ~50 °C.
5. Carefully add 50 mL of the 10× Fe–CAS stock solution to 450 mL NMS agar.
6. Add 5 mL of sterile vitamin stock solution (20 mg/L biotin, 2.0 mg/L folic acid, 5.0 mg/L thiamin HCl, 5.0 mg/L Ca pantothenate, 0.1 mg/L vitamin B_{12}, 5.0 mg/L riboflavin, and 5.0 mg/L nicotinamide; Lidstrom, 1988) to the combined NMS growth agar and Fe–CAS solution.
7. Pour the Fe–CAS/NMS agar into standard petri dishes. After the agar has completely solidified, carefully remove half with a sterilized razor.
8. Prepare 450 mL of NMS growth agar as described in steps 8-10 of Section 2.2 with the appropriate copper concentration. As stated earlier, it is recommended that at least 1 μ*M* of copper be added to the NMS agar as copper limitation may result in repressed growth in some methanotrophic strains. One may prepare NMS agar with and without Fe–EDTA, but the presence of iron in NMS agar will limit siderophore synthesis in most cases. Add 7.5 g Bacto agar to the NMS medium.
9. Carefully pour the NMS agar into the empty space in the agar plate created in step 7 above. The surface of the both the Fe–CAS/NMS agar and NMS agar should be level.
10. Streak methanotroph(s) of interest on the NMS agar half of the plate. Streaking methanotrophs onto the NMS agar only will prevent inhibition of microbial growth by HDTMA as there is no direct contact. It is important that cells be streaked as closely as possible to the boundary of the Fe–CAS/NMS and NMS agars, however, to reduce the time required for any siderophore produced to diffuse into the Fe–CAS/NMS agar.

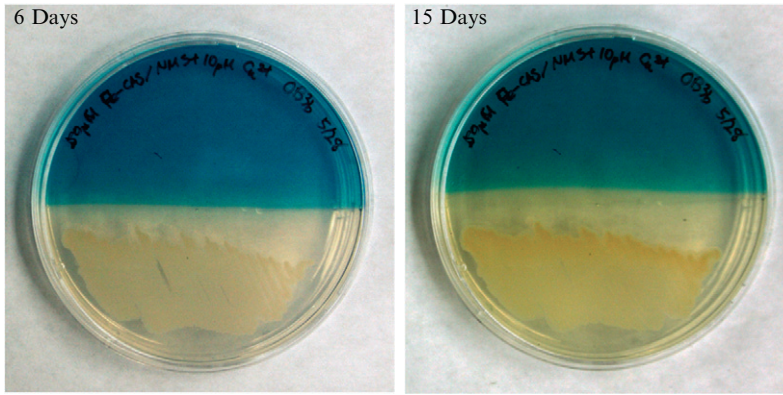

Figure 16.4 An example of the Fe–CAS plate assay performed with *M. trichosporium* OB3b. Ferric iron was present in the NMS agar as Fe–EDTA at a final concentration of 3.8×10^{-4}% (w/v). After 15 days of inoculation, little disappearance of blue color in the Fe–CAS/NMS agar was observed, indicating a small amount of siderophore production.

11. Incubate split Fe–CAS/NMS and NMS agar plates in a sealed container with a 1:1 air-to-methane ratio at the optimal growth temperature of the methanotroph(s) to be tested. A color change from blue to yellowish-orange should begin to appear on Fe–CAS/NMS agar within 2 weeks if a siderophore is produced (Fig.16.4).

4. Conclusions

Here we provide simple instructions for an assay that can be used to screen methanotrophs for chalkophore production. This assay can be modified to screen other cells for chalkophore production by substituting the appropriate growth medium for NMS agar. With this assay, the diversity of microorganisms that produce such copper-specific binding compounds can be more readily determined, and also can be used to help elucidate the genetics of chalkophore synthesis. As other biogenic metal binding compounds, for example, siderophores, can also abstract copper from this assay, parallel studies should be performed with both Cu–CAS and Fe–CAS split plates to determine if and under what conditions cells are producing siderophores that may give a false positive for chalkophore production when using Cu–CAS split plates.

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