Nile Red Detection of Bacterial Hydrocarbons and Ketones in a High-Throughput Format

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ABSTRACT **A method for use in high-throughput screening of bacteria for the production of long-chain hydrocarbons and ketones by monitoring fluorescent light emission in the presence of Nile red is described. Nile red has previously been used to screen for polyhydroxybutyrate (PHB) and fatty acid esters, but this is the first report of screening for recombinant bacteria making hydrocarbons or ketones. The microtiter plate assay was evaluated using wild-type and recombinant strains of *Shewanella oneidensis* and *Escherichia coli* expressing the enzyme OleA, previously shown to initiate hydrocarbon biosynthesis. The strains expressing exogenous *Stenotrophomonas maltophilia oleA*, with increased levels of ketone production as determined by gas chromatography-mass spectrometry, were distinguished with Nile red fluorescence. Confocal microscopy images of *S. oneidensis oleA*-expressing strains stained with Nile red were consistent with a membrane localization of the ketones. This differed from Nile red staining of bacterial PHB or algal lipid droplets that showed intracellular inclusion bodies. These results demonstrated the applicability of Nile red in a high-throughput technique for the detection of bacterial hydrocarbons and ketones.**

IMPORTANCE **In recent years, there has been renewed interest in advanced biofuel sources such as bacterial hydrocarbon production. Previous studies used solvent extraction of bacterial cultures followed by gas chromatography-mass spectrometry (GC-MS) to detect and quantify ketones and hydrocarbons (Beller HR, Goh EB, Keasling JD, Appl. Environ. Microbiol. 76:1212–1223, 2010; Sukovich DJ, Seffernick JL, Richman JE, Gralnick JA, Wackett LP, Appl. Environ. Microbiol. 76:3850–3862, 2010). While these analyses are powerful and accurate, their labor-intensive nature makes them intractable to high-throughput screening; therefore, methods for rapid identification of bacterial strains that are overproducing hydrocarbons are needed. The use of high-throughput evaluation of bacterial and algal hydrophobic molecule production via Nile red fluorescence from lipids and esters was extended in this study to include hydrocarbons and ketones. This work demonstrated accurate, high-throughput detection of high-level bacterial long-chain ketone and hydrocarbon production by screening for increased fluorescence of the hydrophobic dye Nile red.**

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There is intense interest in using biotechnology for the production of fuels and chemicals from renewable resources (1, 2). Current fuels and chemicals derive largely from petroleum hydrocarbon feedstocks that are becoming scarcer and more expensive. Thus, a major goal of commodity biotechnology is to produce hydrocarbons via bacterial metabolism (3). Recent studies have identified the genes involved in cyanobacterial medium-chain alkane biosynthesis (4). Other studies have described bacterial polyolefin biosynthesis via the head-to-head condensation of fatty acyl groups (5–7). While these studies lay the groundwork for future bacterial hydrocarbon production, obtaining bacterial strains suitable for mass production will require rapid screening tools for facilitating the iterative processes of directed microbial engineering and random mutagenesis.

Bioinformatics has revealed approximately 70 bacterial genomes that contain *ole* genes, and a number of those organisms have been demonstrated to produce long-chain hydrocarbons (8). Hydrocarbon biosynthesis has been studied with bacteria of the genus *Shewanella* (7, 9). *Shewanella oneidensis* strain MR-1 produces a single polyolefinic hydrocarbon, 3,6,9,12,15,19,22,25,28-hentriacontanonaene, and contains the *oleABCD* genes. The *ole* gene cluster was shown to be essential for polyolefin biosynthesis (10), and the isolated expression of *oleA*, which encodes a fatty acyl-coenzyme A (CoA) condensase, produces a long-chain ketone in *S. oneidensis*. Additionally, experiments with a strain of *S. oneidensis* MR-1 showing a deletion of the *ole* genes suggested that the polyolefin may be involved in adaption to lowered temperature (7). Foreign *oleA* genes expressed in *S. oneidensis* result in the production of a variety of ketones, depending on the source. For example, the expression of *oleA* from *Stenotrophomonas maltophilia* resulted in the production of more than 15 ketones derived from the condensation of different fatty acids (7). The long-chain ketones, ranging from 26 to 30 carbons in length, are of interest as fuel precursors or novel chemicals.

Chen et al. (11) previously described a high-throughput method using Nile red to detect neutral lipids in microalgae. The present study describes a similar high-throughput method for identification of mutant strains of *S. oneidensis* and *Escherichia coli* that produce higher levels of hydrocarbons or ketones. While nonfluorescent in water, Nile red fluoresces in a hydrophobic environment (12). Nile red has previously been used to screen bacteria, cyanobacteria, haloarchaea, and microalgae for those producing fatty acids and esters (11, 13–20). Nile red has also been used to screen for isoprenoid-derived hydrocarbon production in microalgae (21) and for triacylglyceride production in *Saccharomyces cerevisiae* (22). Previous studies typically used agar plates with Nile red in the medium (14–18, 23). In those examples, strains making more fatty acids or esters could be identified using fluorescence microscopic techniques. In initial agar plate studies, both wild-type *S. oneidensis* and a derivative expressing *S. maltophilia oleA* were compared to the non-hydrocarbon-producing *oleABCD* deletion (Δ*oleABCD*) strain (see Table S1 in the supplemental material). On plates, the three strains showed a low level of Nile red fluorescence that could not be differentiated (data not shown). Unlike for the fatty esters, which comprise up to 80% of the cell mass and are typically deposited in cellular granules, it is possible that the level of hydrocarbon production in these *Shewanella* strains may be too low to differentiate on plates.

Because colony fluorescence could not be differentiated, subsequent studies were conducted with liquid culture to test Nile red for use as a high-throughput screening vehicle. Stationary-phase liquid cultures (Luria broth [LB]) of *S. oneidensis* expressing *S. maltophilia oleA* on a pBBAD vector, *S. oneidensis* with an empty pBBAD vector, and the Δ*oleABCD* strain with an empty pBBAD vector were grown in microtiter plates with LB-kanamycin (50 μg/ml). Nile red in dimethyl sulfoxide (DMSO) (1 mg/ml) was added to give a final concentration of 1 μg/ml. Expression of *oleA* under the control of the *araBAD* promoter was induced with 0.2% arabinose at an optical density at 600 nm (OD_{600}) of 0.5 to 0.8, and strains harboring empty vectors were mock induced. The plates were incubated overnight at 30°C in a microtiter plate orbital shaker with 70% humidity (optimization of dye contact time is described in Fig. S1 in the supplemental material). To minimize photobleaching of the dye, the plates were incubated in the dark. Fluorescence intensity was measured with a fluorescence spectrophotometer (Molecular Devices SpectraMax M2) using an excitation wavelength of 580 nm and an emission wavelength of 640 nm. Percentages of the Nile red fluorescence signal relative to that of *S. oneidensis* with the empty pBBAD vector, and corrected for the optical density of the cultures (relative fluorescence units [RFU]/OD_{600} unit), are reported for each strain, and standard deviations are shown for triplicate samples (Fig. 1A). The Nile red signal for *S. oneidensis* that is expressing *S. maltophilia oleA* is significantly increased over the background signal for both *S. oneidensis* and the Δ*oleABCD* strain (analysis of variance [ANOVA]; $P = 0.0164$). These results are in agreement with results for gas chromatography with flame ionization detection (GC-FID) analysis of hydrocarbon and ketone production performed using a modified Bligh and Dyer method as described in the supplemental material (Fig. 1B; see also Fig. S3 in the supplemental material) (24). These results suggested that Nile red fluorescence is a good indicator of increased long-chain hydrocarbon or ketone production, detectable above the background stain of membrane components.

Analogous experiments were carried out using BODIPY 493/503 (Invitrogen), as similar dyes have also been used for hydrophobic-molecule screening (25). The methods and results are reported in the supplemental material (see Fig. S2 in the supplemental material). BODIPY was found not to be as sensitive to changes in hydrocarbon and ketone production as Nile red. This may be a result of the smaller separation between the excitation and emission maximum wavelengths than for Nile red. Furthermore, BODIPY 493/503 is considerably more expensive and more sensitive to photobleaching than Nile red (26). For these reasons, Nile red was chosen for further study.

To examine whether Nile red fluorescence could also be used as a method for detecting increased hydrocarbon production in *E. coli*, we analyzed Nile red fluorescence of *E. coli* WM3064 harboring either *S. maltophilia oleA* on pBBAD or an empty pBBAD vector. The *E. coli* cultures were induced and assayed as described above for *Shewanella*, with the exception that the cells were grown at 37°C in the presence of diaminopimelic acid (DAP) since WM3064 is a DAP auxotroph. The Nile red signal was significantly (ANOVA; $P = 0.0004$) higher in *E. coli* expressing *oleA* (Fig. 1C). These Nile red data are in agreement with GC-FID analysis in which ketone production was observed in the strain expressing *oleA* but not in the strain carrying an empty vector (Fig. 1D; see also Fig. S4 in the supplemental material). Together with the results for *Shewanella*, these data demonstrated that the fluorescent dye Nile red could be used to identify bacterial strains producing long-chain hydrocarbons or ketones in a microtiter plate format.

To test the feasibility of using Nile red in a high-throughput screen, 96 colonies of a 1:1:1 mixed population of wild-type *S. oneidensis*, *S. oneidensis* expressing *S. maltophilia oleA*, and the Δ*oleABCD* strain were screened with Nile red as described above. A distribution of Nile red fluorescence signals ranging from 800 to 150 RFU/OD_{600} unit was observed (Fig. 1E). Extracts from the five colonies with the highest and lowest Nile red fluorescence levels were analyzed by GC-FID to identify each strain by its previously determined hydrocarbon or ketone profile. Of the colonies giving the five highest Nile red signals, all five produced increased amounts of ketones, indicating that all five were the strains expressing the *S. maltophilia oleA* gene. The five colonies with the lowest Nile red fluorescence levels either produced the single hydrocarbon signature of MR-1 or did not produce the hydrocarbon, as previously observed in the Δ*oleABCD* strain (Fig. 1E, insets; see also Fig. S5 in the supplemental material). These results are consistent with the previous observation that both the wild-type and the Δ*oleABCD* strains gave background levels of Nile red fluorescence.

Unlike polyhydroxyalkanoates (PHAs), which accumulate in granules inside the cells, the hydrocarbon and ketone products of the *ole* genes are thought to partition into the membrane; however, this has not been investigated. With evidence that Nile red is capable of detecting increased ketone production *in vivo*, Nile red staining of ketone-producing strains was used to gain information regarding the cellular localization of the ketones using fluorescence microscopy. A ketone producer strain of *S. oneidensis* expressing *S. maltophilia oleA* was incubated with 2 μg/ml Nile red and 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI), a DNA dye, for at least 15 minutes. These cells were adhered to microscope coverslips, mounted on glass slides, and visualized with a Nikon C1si laser scanning confocal microscope (Fig. 2). Cells that have been imaged as a cross section appeared as a ring of Nile red (Fig. 2A). These same cells showed concentrated blue foci of the

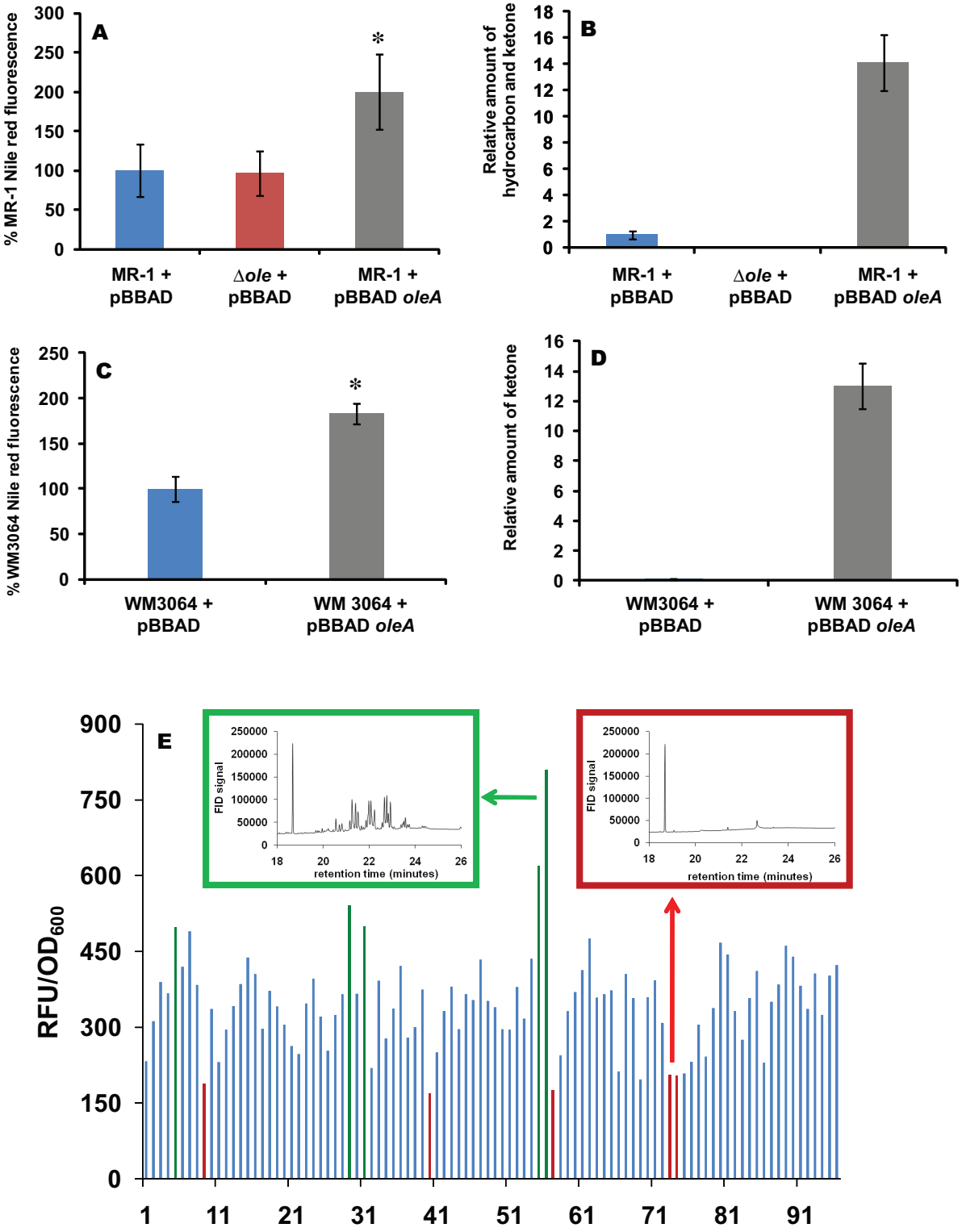

FIG 1 (A) Percentages of MR-1 Nile red fluorescence normalized to optical density (OD_{600}) for *S. oneidensis* MR-1 with empty pBBAD (blue), the Δ*ole* strain with empty pBBAD (red), and *S. oneidensis* with *S. maltophilia oleA* in pBBAD (gray). An asterisk denotes a significantly different strain (ANOVA; $P = 0.0164$). (B) GC-FID amounts for strains listed in panel A relative to the level for a 12-tricosanone standard normalized to optical density (OD_{600}) and to the amount produced by *S. oneidensis* with empty pBBAD. (C) Percentages of Nile red fluorescence normalized to optical density (OD_{600}) for *E. coli* with empty pBBAD (blue) and *E. coli* with *S. maltophilia oleA* in pBBAD (gray). An asterisk denotes a significantly different strain (ANOVA; $P = 0.0004$). (D) GC-FID amounts for strains listed in panel C relative to the level for a 12-tricosanone standard normalized to optical density (OD_{600}) and to the amount produced by *S. oneidensis* with empty pBBAD. (E) Relative Nile red fluorescence normalized to the optical densities (RFU/OD_{600} unit) of 96 colonies of a 1:1:1 mixture of the *S. oneidensis* strains listed in panel A. The five highest and the five lowest Nile red signals are denoted by green and red bars, respectively. The GC-FID chromatograms from the colonies producing the highest (green) and lowest (red) Nile red signals are inset.

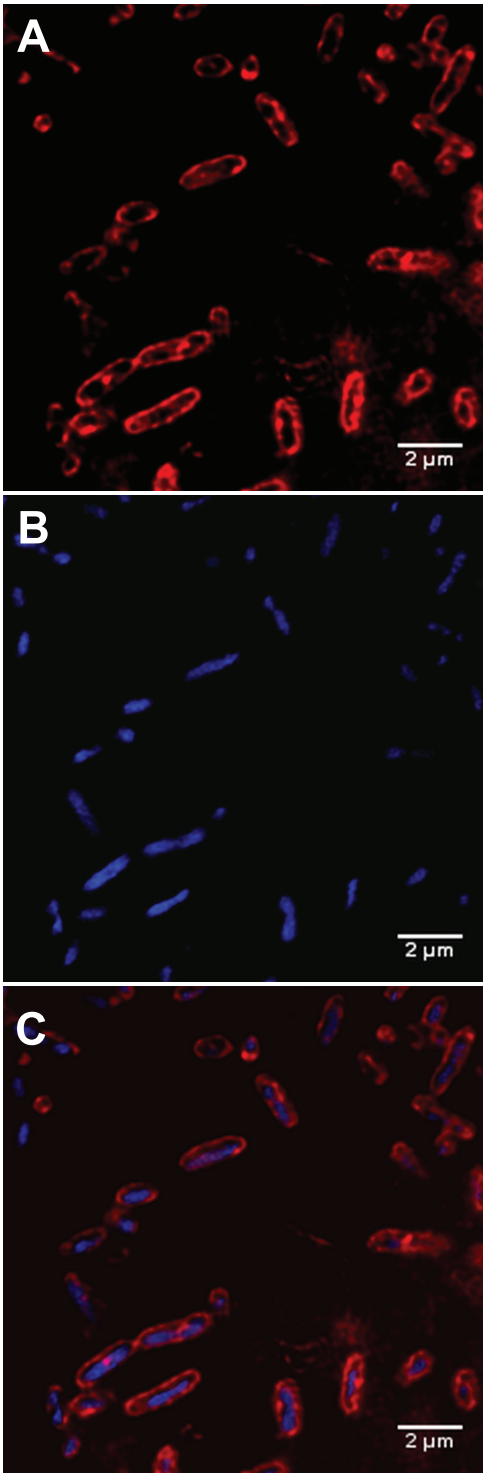

FIG 2 Confocal fluorescence micrographs of a ketone producer strain of *S. oneidensis* expressing *S. maltophilia oleA*. (A) Nile red fluorescence; (B) DAPI fluorescence; and (C) merged image of Nile red and DAPI.

DAPI stain (Fig. 2B). In the merged image, the Nile red membrane stain surrounded the DAPI nuclear stain (Fig. 2C). These results are consistent with a localization of the ketones and hydrocarbons in cellular membranes and perhaps in some extracellular hydrophobic material.

Implications. These results showed that Nile red fluorescence could be used to identify bacterial strains with increased levels of olefinic hydrocarbons or ketones that derive from the head-to-head hydrocarbon biosynthetic pathways. Academic and industrial investigators are researching the production of alkanes or sesquiterpenoid hydrocarbons. It is likely that the methods described here will prove generally useful for high-throughput screening of bacteria to identify strains that are useful for mass production of hydrocarbons or ketones.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00109-11/-/DCSupplemental.

Text S1, DOC file, 0.032 MB.
Table S1, DOC file, 0.044 MB.
Figure S1, DOC file, 0.032 MB.
Figure S2, DOC file, 0.033 MB.
Figure S3, DOC file, 0.125 MB.
Figure S4, DOC file, 0.621 MB.
Figure S5, PDF file, 0.419 MB.

REFERENCES

1. **Steen EJ, et al.** 2010. Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. Nature **463**:559–562.
2. **Keasling JD.** 2010. Manufacturing molecules through metabolic engineering. Science **330**:1355–1358.
3. **Regalbuto JR.** 2009. Engineering. Cellulosic biofuels—got gasoline? Science **325**:822–824.
4. **Schirmer A, Rude MA, Li X, Popova E, del Cardayre SB.** 2010. Microbial biosynthesis of alkanes. Science **329**:559–562.
5. **Beller HR, Goh EB, Keasling JD.** 2010. Genes involved in long-chain alkene biosynthesis in *Micrococcus luteus*. Appl. Environ. Microbiol. **76**: 1212–1223.
6. **Friedman L, Rude M.** September 2008. Production of fuels derived from recombinant microorganisms. WO patent 2008113041.
7. **Sukovich DJ, et al.** 2010. Structure, function, and insights into the biosynthesis of a head-to-head hydrocarbon in *Shewanella oneidensis* strain MR-1. Appl. Environ. Microbiol. **76**:3842–3849.
8. **Sukovich DJ, Seffernick JL, Richman JE, Gralnick JA, Wackett LP.** 2010. Widespread head-to-head hydrocarbon biosynthesis in bacteria and role of OleA. Appl. Environ. Microbiol. **76**:3850–3862.
9. **Sugihara S, et al.** 2010. Possible biosynthetic pathways for all cis-3,6,9,12,15,19,22,25,28-hentriacontanonaene in bacteria. Lipids **45**: 167–177.
10. **Frias JA, Richman JE, Erickson JS, Wackett LP.** 2011. Purification and

characterization of OleA from *Xanthomonas campestris* and demonstration of a non-decarboxylative Claisen condensation reaction. J. Biol. Chem. **286**:10930–10938.
11. **Chen W, Zhang C, Song L, Sommerfeld M, Hu Q.** 2009. A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. J. Microbiol. Methods **77**:41–47.
12. **Greenspan P, Mayer EP, Fowler SD.** 1985. Nile red: a selective fluorescent stain for intracellular lipid droplets. J. Cell Biol. **100**:965–973.
13. **Manilla-Pérez E, et al.** 2010. Isolation and characterization of a mutant of the marine bacterium *Alcanivorax borkumensis* SK2 defective in lipid biosynthesis. Appl. Environ. Microbiol. **76**:2884–2894.
14. **Manilla-Perez E, et al.** 2010. Analysis of lipid export in hydrocarbonoclastic bacteria of the genus *Alcanivorax*: identification of lipid export-negative mutants of *Alcanivorax borkumensis* SK2 and *Alcanivorax jadensis* T9. J. Bacteriol. **192**:643–656.
15. **Shrivastav A, et al.** 2010. Isolation of promising bacterial strains from soil and marine environment for polyhydroxyalkanoates (PHAs) production utilizing *Jatropha* biodiesel byproduct. Int. J. Biol. Macromol. **47**:283–287.
16. **Berlanga M, Montero MT, Fernández-Borrell J, Guerrero R.** 2006. Rapid spectrofluorometric screening of poly-hydroxyalkanoate-producing bacteria from microbial mats. Int. Microbiol. **9**:95–102.
17. **Kranz RG, Gabbert KK, Madigan MT.** 1997. Positive selection systems for discovery of novel polyester biosynthesis genes based on fatty acid detoxification. Appl. Environ. Microbiol. **63**:3010–3013.
18. **Legat A, Gruber C, Zangger K, Wanner G, Stan-Lotter H.** 2010. Identification of polyhydroxyalkanoates in *Halococcus* and other haloarchaeal species. Appl. Microbiol. Biotechnol. **87**:1119–1127.
19. **da Silva TL, Reis A, Medeiros R, Oliveira AC, Gouveia L.** 2009. Oil production towards biofuel from autotrophic microalgae semicontinuous cultivations monitorized by flow cytometry. Appl. Biochem. Biotechnol. **159**:568–578.
20. **Tyo KE, Zhou H, Stephanopoulos GN.** 2006. High-throughput screen for poly-3-hydroxybutyrate in *Escherichia coli* and *Synechocystis* sp. strain PCC6803. Appl. Environ. Microbiol. **72**:3412–3417.
21. **Weiss TL, et al.** 2010. Raman spectroscopy analysis of botryococcene hydrocarbons from the green microalga *Botryococcus braunii*. J. Biol. Chem. **285**:32458–32466.
22. **Siloto RM, Truksa M, He X, McKeon T, Weselake RJ.** 2009. Simple methods to detect triacylglycerol biosynthesis in a yeast-based recombinant system. Lipids **44**:963–973.
23. **Amara AA, Steinbüchel A, Rehm BH.** 2002. In vivo evolution of the *Aeromonas punctata* polyhydroxyalkanoate (PHA) synthase: isolation and characterization of modified PHA synthases with enhanced activity. Appl. Microbiol. Biotechnol. **59**:477–482.
24. **Bligh EG, Dyer WJ.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911–917.
25. **Cooper MS, Hardin WR, Petersen TW, Cattolico RA.** 2010. Visualizing "green oil" in live algal cells. J. Biosci. Bioeng. **109**:198–201.
26. **Listenberger LL, Brown DA.** 2007. Fluorescent detection of lipid droplets and associated proteins. Curr. Protoc. Cell Biol. **Chapter 24**:Unit 24.2.