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Research Paper

Monitoring succinoglycan production in single *Sinorhizobium meliloti* cells by Calcofluor white M2R staining and time-lapse microscopy

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ABSTRACT

Here, we describe a simple, non-time consuming and inexpensive method for monitoring of Calcofluor white M2R-binding exopolysaccharides in individual bacterial cells. This method was demonstrated by time-lapse microscopy of succinoglycan-producing cells of the plant-symbiotic alpha-proteobacterium *Sinorhizobium meliloti*. The method is most likely applicable to other bacteria producing β-(1 → 3) and β-(1 → 4) linked polysaccharides.

1. Introduction

Fluorescent dyes have been applied to visualize living cells for over one hundred years (Provazec, 1914). Early studies demonstrated the advantage of using fluorescent non-toxic compounds for staining of growing cells. A widely used fluorescent dye is Calcofluor White M2R (CF). Due to its cellulose and chitin binding properties, CF was initially used to stain cell walls of plants and fungal hyphae (Darken, 1962) but became later generally regarded as a fluorescent dye for staining β-(1 → 3) and β-(1 → 4) linked polysaccharides (Maeda & Ishida, 1967). The binding of CF to the corresponding glycan produces intense light blue or bright violet-blue coloration under violet or UV excitation, respectively. More recently, a CF-based method was reported for chitin identification in fossil specimens (Ehrlich et al., 2013). This method also allowed the detection of this polysaccharide for the first time in sponges and corals (Bo et al., 2012; Ehrlich et al., 2017). In addition to the aforementioned applications, CF has also proved to be applicable to visualizing bacterial polysaccharide-containing biofilms (Chen, Lee, Yang, Peng, & Lai, 2006; Kim & Wei, 2009; Thurnheer, Gmür, & Guggenheim, 2004). Polysaccharides are important cell-surface polymers serving diverse functions in bacteria (Limoli, Jones, & Wozniak, 2015; Nwodo, Green, & Okoh, 2012).

The plant-symbiotic α-proteobacterium *Sinorhizobium meliloti* produces the exopolysaccharide succinoglycan (or EPS I) which is a polymer composed of octasaccharide repeating units. Each repeating unit contains one galactose and seven glucose molecules joined by β-(1 → 4), β-(1 → 3), and β-(1 → 6) glycosidic linkages. It can be decorated by acetyl, succinyl, and pyruvyl groups (Åman, McNeil, Franzén, Darvill, & Albersheim, 1981; Reinhold et al., 1994). Succinoglycan is produced in two molecular forms, one of high molecular weight (consisting of hundreds of the repetitive octasaccharide subunit) and the other of low molecular weight (consisting of monomers, dimers, and trimers of the repetitive octasaccharide subunit). Succinoglycan produced by *S. meliloti* is a key determinant for the establishment of an effective symbiosis with *Medicago sativa* plants. Specifically, succinoglycan is required to promote infection of alfalfa root nodules (Leigh, Signer, & Walker, 1985). *S. meliloti exoY* mutants, producing no succinoglycan, fail in nodule invasion (Cheng & Walker, 1998). Other functions including protection against environmental stress, host defense, attachment to abiotic and biotic surfaces, and signaling, have also been attributed to this exopolysaccharide (Downie, 2010).

From the industrial point of view, succinoglycan possess rheological properties similar to those of xanthan. Furthermore, it has been shown that this polysaccharide is more stable to temperature, to acid and basic pHs and saline conditions than the xanthan. Succinoglycan is used as thickener in detergents, in toilet bowl cleaners, in frozen food or feedstuff products, in biocide formulations, among others (BeMiller & Whistler, 2012; Dumitriu, 2004; Williams & Phillips, 2012)

In contrast to the surface polysaccharides produced by *Escherichia coli* and other members of the *Enterobacteriaceae* family, polysaccharides produced by *S. meliloti*, with the exception of a Kdo-rich polysaccharide (Petrovics et al., 1993), are non-immunogenic. This lack

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of immunoreactivity complicates generation of specific antibodies for studying biosynthesis and export of surface polysaccharides in single *S. meliloti* cells.

In addition to the aforementioned applications, CF has also been used as a qualitative dye to visualize succinoglycan-producing *S. meliloti* colonies (Leigh et al., 1985). However, not all bacterial polysaccharides bind to CF, restricting thereby its application to certain types of polymers.

Here, we describe a simple and reliable method based on the use of CF and time-lapse microscopy to monitor production and extracellular accumulation of succinoglycan at single cell level in *S. meliloti*.

2. Hypothesis

The binding specificity of Calcofluor white for succinoglycan allows the detection and monitoring of the production of this polysaccharide in single cells of *Sinorhizobium meliloti*.

3. Materials and methods

Calcofluor White M2R (Fluorescent brightener 28, Sigma Aldrich, St. Louis, Missouri, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich, St. Louis, Missouri, USA) at a concentration of 50 mg/mL.

S. meliloti wild type strain 2011 and its *exoY-lacZ/aacC*1 mutant RmAR9007 (Keller et al., 1995) were grown in GMS (Zevenhuizen & van Neerven, 1983) medium at 28 °C. When required, antibiotics were added at the following concentrations: 10 μg/mL nalidixic acid, 40 μg/mL gentamicin.

Production of succinoglycan by *S. meliloti* strains was demonstrated by the fluorescence of the colonies growing on GMS agar plates supplemented with 200 μg/mL of CF (Leigh et al., 1985). Plates were incubated for 3 days at 28 °C and then examined under UV light.

For microscopic observations, overnight bacterial cultures, grown in GMS medium, were diluted 1/100 with fresh GMS medium containing CF 50 μg/mL and incubated at 28 °C with shaking at 200 rpm. When cultures reached the stationary growth phase (optical density at 600 nm (OD600) of 1.5), 2.5 μL of the culture were deposited onto pads (In situ adhesive frames 1.7 × 2.8 cm, PEQLAB GmbH, Erlangen, Germany) containing a thin layer of 1% of agarose (Molecular biology grade agarose, EUROGENTEC S.A., Seraing, Liège, Belgium) in GMS. Pads were covered with glass coverslips and immediately observed by microscopy.

For time-lapse analysis, the *S. meliloti* wild type strain was grown as mentioned above in GMS broth without CF. When culture reached the exponential phase of growth (OD600 of 0.3), 1 mL of the culture was centrifuged at 1500*g* for 3 min. The supernatant was removed and remaining cells were washed twice with phosphate-buffered saline (PBS) and finally suspended in 1 mL of PBS. A 2.5 μL aliquot of the cell suspension (2.5×10^5 CFU) was deposited onto pads containing a thin layer of 1% of agarose in GMS supplemented with CF 50 μg/mL. Pads were sealed by placing the glass coverslips over the adhesive surface of the pads and maintained at 28 °C for 2 h, in Petri dishes containing a wet filter paper, before time-lapse microscopy. Images were acquired every 10 min at 28 °C.

Permeabilization of *S. meliloti* cells was carried out by using chloroform. 100 μL aliquots of *S. meliloti* cultures grown till the stationary growth phase were centrifuged at 1500*g* for 3 min. Supernatants were removed and remaining cells were carefully washed twice with PBS to remove any cell-attached succinoglycan. Cells were suspended in 100 μL of PBS containing CF (final concentration 50 μg/mL) and immediately permeabilized by adding 20 μL chloroform. The resulting suspension was vortexed twice for 10 s and incubated for 10 min at room temperature (Åmes, Prody, & Kustu, 1984). After incubation, 2.5 μL of the cell suspension (upper phase) were deposited onto pads covered with 1% of agarose in GMS and observed under the microscope.

In all cases, bacteria were visually examined by differential interference contrast and epifluorescence microscopy using a Nikon Eclipse Ti-E (Nikon Instruments Inc., Melville, New York) equipped with 100 x CFI Apo TIRF Oil objective (numerical aperture of 1.49) with AHHC FL filterset F36-513 DAPI (excitation band pass 387/11 nm, beam splitter 409 nm, emission band pass 447/60 nm).

Images were acquired with an Andor iXon3 885 EMCCD camera (Andor Technology Ltd., Belfast, UK). Exposure times for fluorescence images ranged from 50 to 500 ms. Image acquisition and adjustment were done with Nikon NIS elements 4.0 software. Images were processed with ImageJ (Schneider, Rasband, & Eliceiri, 2012).

4. Results and discussion

To validate the method, the succinoglycan-producing wild type strain *S. meliloti* 2011, was used (Becker et al., 2002; González, York, & Walker, 1996). Mutant strain RmAR9007 (a derivative of *S. meliloti* 2011, *exoY:lacZ*/Gm) served as negative control for succinoglycan production. This mutant is unable to produce succinoglycan since it lacks the ExoY galactosyl transferase catalysing the initial step of octasaccharide subunit synthesis (Reuber & Walker, 1993). First, the CF phenotypes of colonies from wild type strain *S. meliloti* 2011 and *exoY* mutant RmAR9007 were tested. Both strains were grown in GMS agar supplemented with CF at the recommended concentration of 200 μg/mL (Leigh et al., 1985). After 3 days of growth, colonies formed by the wild type strain were fluorescent when observed under UV light. In contrast, colonies from the *exoY* mutant strain RmAR9007, did not fluoresce under UV light (Fig. 1).

As reported by Maeda and Ishida (1967), CF is highly specific for polysaccharides containing β-(1 → 3) and β-(1 → 4) linkages. In the particular case of *S. meliloti*, which produces at least four known polysaccharides −capsular polysaccharide, succinoglycan (EPS I), galactoglucan (EPS II) and lipopolysaccharide– the only polysaccharide capable of binding to CF, and hence mediating fluorescence, is succinoglycan (Finan et al., 1985; Leigh, Reed, Hanks, Hirsch, & Walker, 1987; Leigh et al., 1985). More recently reports about the use of CF to demonstrate the production of succinoglycan in *S. meliloti*, indicated that the CF fluorescence is affected by medium acidification (Geddes, González, & Oresnik, 2014; Hawkins, Geddes, & Oresnik, 2017).

Taking advantage of the affinity of CF to succinoglycan, we tested whether a lower concentration of the dye is appropriate for microscopic visualization of *S. meliloti* cells producing succinoglycan. To this end, *S. meliloti* wild type and RmAR9007 strains were grown in GMS broth supplemented with 50 μg/mL of CF. At the stationary growth phase (OD600 of 1.5), samples of 2.5 μL were taken and deposited onto GMS agarose pads (see materials and methods) and observed by epifluorescence microscopy.

Wild type cells cultured without CF did not show any signal when observed by epifluorescence microscopy with the filter for DAPI (Fig. 2A). In contrast, a few cells (~ 13%) from cultures of the wild type strain grown in presence of CF showed fluorescent signals at the cell poles or throughout the cell (Fig. 2B). Although the cellular site of

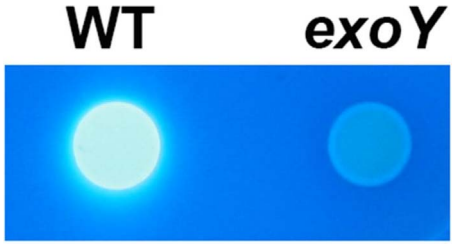

Fig. 1. Calcofluor phenotypes of colonies from *S. meliloti* wild type 2011 (WT) and *exoY* mutant RmAR9007 deficient in succinoglycan production (*exoY*).

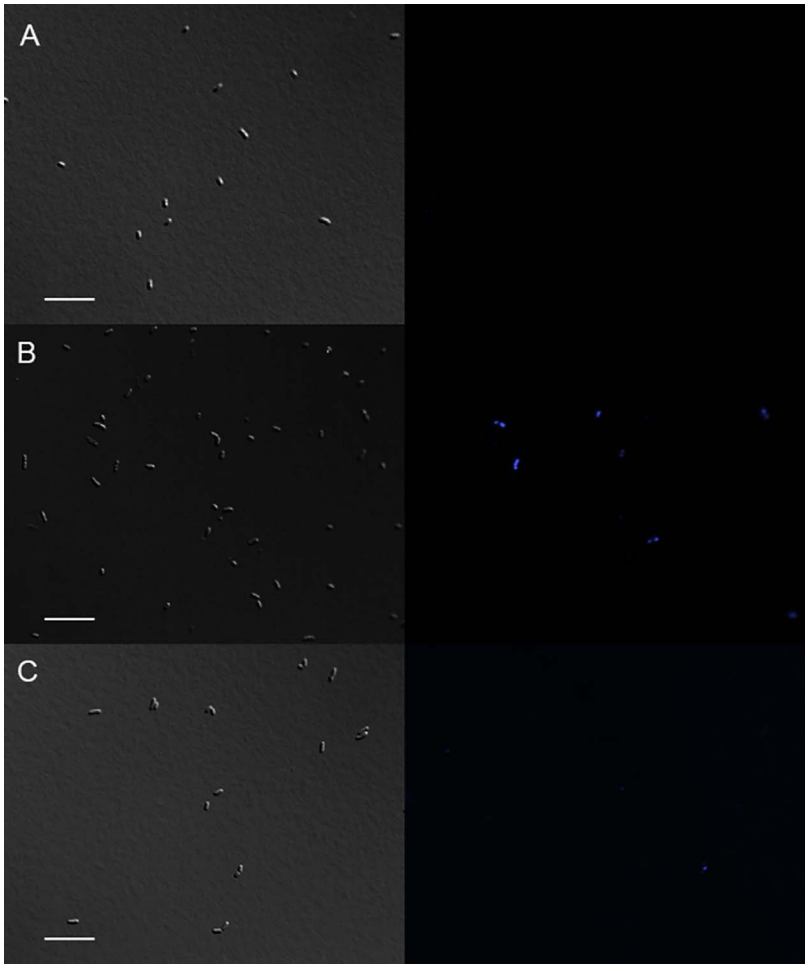

Fig. 2. Differential interference contrast (left column) and CF fluorescence (right column) microscopy of *S. meliloti* stationary phase cells grown in GMS containing CF.(A) *S. meliloti* 2011 wild type cells grown in GMS without CF; (B) *S. meliloti* 2011 wild type cells grown in GMS with 50 μg/mL of CF; (C) *S. meliloti* RmAR9007 cells grown in GMS with 50 μg/mL of CF. Scale bars indicate 10 μm.

succinoglycan export in *S. meliloti* is unknown, the fact that incipient fluorescent signals were detected at cell poles suggests that secretion of this polymer may occur at cell poles.

As expected, *exoY* mutant cells, which do not produce succinoglycan, were not fluorescent (Fig. 2C). We also tested 100 μg/mL of CF (data not shown), but as no differences were observed, neither in fluorescence intensity nor in fluorescence background using 50 or 100 μg/mL of CF, we decided hereafter to use the lowest tested concentration.

The fact that *exoY* mutant cells, which are only affected in succinoglycan biosynthesis, do not show fluorescence supports that the only cell surface component of *S. meliloti* capable of binding to CF was succinoglycan.

We wondered whether the limited number of wild type cells showing fluorescence was the consequence of the release of succinoglycan from cells (and subsequent diffusion into the medium) or of an insufficient time of culturing for polymer production. To answer this question, we performed time-lapse microscopy analysis of *S. meliloti* wild type cells growing on GMS agarose pads supplemented with CF. Prior to inoculation of the agarose pads, cells cultured till the exponential growth phase were washed with PBS in order to remove any cell-attached succinoglycan. To account for a possible lag phase of growth after transferring cells from liquid culture to the agarose pads, the pads were incubated for 2 h at 28 °C prior to the time-lapse analysis.

Images were taken every 10 min over 10 h. Fig. 3A shows representative images of this time course at time points where the production of succinoglycan by *S. meliloti* cells became evident. A close-up view of representative cells is shown in Fig. 3B. The complete time-lapse experiment is shown in supplementary video 1.

At time 0 (immediately after 2 h of incubation) only two cells showing fluorescent signals (~ 5% of cell population) were detected. Thirty min later the number increased to six (~17%) and at 60 min, ~ 48% of the cell population showed fluorescent signals. This percentage further increased, reaching ~ 70% at 180 min (Fig. 3A). It is worth mentioning that production of succinoglycan in *S. meliloti* cells grown on the GMS agarose pads even started before cell divisions were observed.

At 280 min nearly 100% of the cell population produced succinoglycan at diverse levels, as suggested by the intensity of the fluorescence surrounding the cells (supplementary video 1).

These results demonstrate that CF staining combined with time-lapse microscopy is a very sensitive method for detection of succinoglycan, even at single cell level.

Although fluorescence intensity of CF-stained cells can be used for relative quantification of succinoglycan (Fields et al., 2012), accurate quantification is limited at single cell level. Moreover, diffusion of EPS I results quickly in spreading of succinoglycan produced by a single cell over the agarose medium pads.

In order to determine whether this method is also applicable to visualize intracellular succinoglycan, we performed an assay in which cellular uptake of CF was facilitated by chloroform permeabilization of *S. meliloti* cells. To this end, stationary phase wild type and RmAR9007 mutant cells were washed, permeabilized with chloroform in presence of CF and observed under the microscope.

Non-permeabilized wild type cells showed scattered, non-cell associated fluorescent signals, suggesting insufficient cell washing and incomplete removal of cell-attached succinoglycan (supplementary Fig. 1A). On the contrary, non-permeabilized cells of mutant

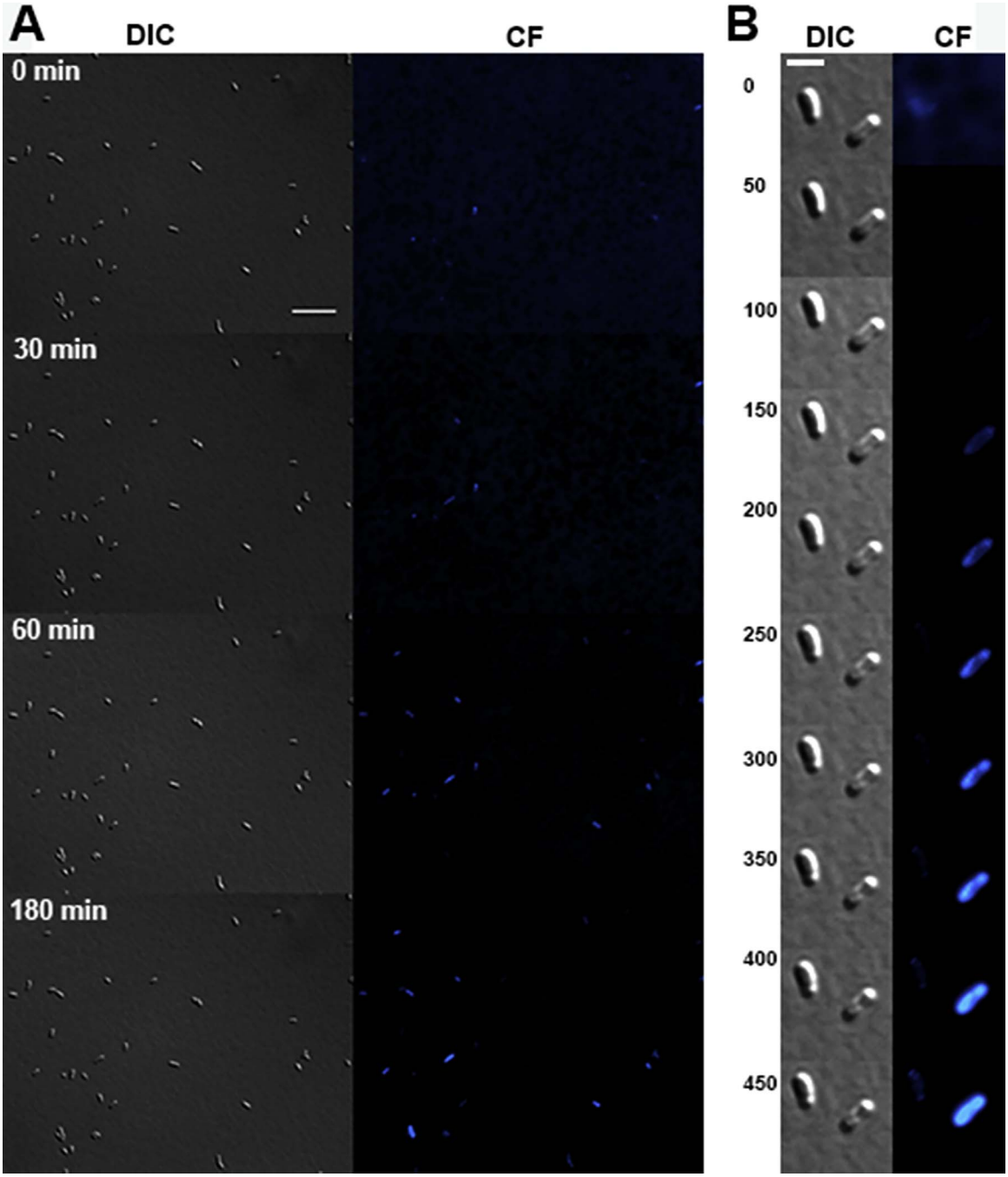

Fig. 3. (A) Representative images from a time-lapse microscopy analysis of succinoglycan production by *S. meliloti* cells growing on GMS agarose supplemented with CF. The time of image acquisition is indicated in the upper left corner of each image. Left columns show differential interference contrast images (DIC), right columns represent CF fluorescence images (CF). Scale bar indicates 10 μm.(B) Close-up view of representative cells from Fig. 3A (dotted square in Fig. 3A indicates the magnified cells). The time (min) of image acquisition is shown to the left of each image. Left columns show differential interference contrast images (DIC), right columns represent epifluorescence images (DAPI). Scale bar indicates 2 μm. The complete time lapse in shown in Supplementary video 1.

RmAR9007, did not show any fluorescent signals (supplementary Fig. 1C).

Fluorescent signals were detected in all permeabilized wild type cells (supplementary Fig. 1B). Unexpectedly, permeabilized cells from mutant RmAR9007 were fluorescent (supplementary Fig. 1D). Considering that this mutant strain is affected in the initial steps of the synthesis of the octasaccharide-repeating unit of succinoglycan, no intracellular succinoglycan is expected to be present. Therefore, the fluorescent signals observed within the permeabilized RmAR9007 mutant cells indicate that, in addition to succinoglycan, other intracellular components of *S. meliloti* bind to CF. Although, the identity of these components remains to be determined, we speculate that intermediates of the peptidoglycan biosynthesis pathway, such as glycan chains of N-acetylglucosamine and N-acetylmuramic acid residues which are linked by β 1- 4 bonds, may bind to CF (Barreteau et al., 2008).

Recently, a new novel linear mixed-linkage (1 → 3)(1 → 4)-β-glucan was reported to be synthetized by *S. meliloti*, but its production was only observed upon artificially increasing the levels of cyclic di-guanylate (Pérez-Mendoza et al., 2015).

Due to the interference with other intracellular components, usage of CF for visualizing intracellular succinoglycan in permeabilized cells

of *S. meliloti* is not recommended.

5. Conclusion

The method presented here, based on the use of CF followed by time-lapse microscopy analysis, allows visualization and monitoring of the production of succinoglycan in *S. meliloti* cells. Taking into consideration that so far no alternative method for visualizing the production of this exopolysaccharide in individual cells has been described, staining by CF is a promising option. It showed several desirable features such as: the fluorescence stability, since no photobleaching was observed even after exposing CF-stained cells every 10 min over 10 h; the high sensitivity, even for detection of low quantities of succinoglycan such as those produced by single cells; almost null background fluorescence; and once the bacterial cultures have grown, the procedure is timesaving. Not less important the entire procedure is not expensive.

Moreover, CF detection could also be combined with monitoring of fluorescent proteins, such as mCherry and/or GFP to determine the localization of fusions of these fluorescent reporters to proteins associated to the biosynthesis and export of this particular polysaccharide. This protocol is most likely applicable to other bacteria producing exopolysaccharides containing β-(1 → 3) and β-(1 → 4) linkages.

Author contributions

E.J. conceived and designed the methodology and experiments. E.J. and A.B. were responsible for funding acquisition and project management. E.J., J.L., and D.M. performed the experiments and collected the data. J.L., and D.M. analyzed the data. E.J. and A.B. wrote the paper.

Competing interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2017.11.059.

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