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Common Dyes Used to Determine Bacterial Polysaccharides on Agar Are Affected by Medium
Acidification

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Abstract

In this work we highlight effects of pH on bacterial phenotypes when using the bacteriological dyes Aniline blue, Congo red, and Calcofluor white to analyze polysaccharide production. Study of galactose catabolism in *Sinorhizobium meliloti* led to the isolation of a mutation in *dgok1*, which was observed to overproduce exopolysaccharides when grown in the presence of galactose. When this mutant strain was spotted onto plates containing Aniline blue, Congo red, or Calcofluor white, the intensity of the associated staining was strikingly different when compared to the Wild-type. Additionally, a Calcofluor dull phenotype was observed, suggesting production of a polysaccharide other than succinoglycan. Further investigation of this phenotype revealed that these results were dependent on medium acidification, as buffering at pH 6 had no effect on these phenotypes, while medium buffered at pH 6.5 resulted in a reversal of the phenotypes. Screening for mutants of the *dgok1* strain that were negative for the aniline blue phenotype yielded a strain carrying a mutation in *tkt2* which is annotated as a putative transketolase. Consistent with the plate phenotypes, when this mutant was grown in broth cultures it did not acidify its growth medium. Overall this work shows that caution should be exercised in evaluating polysaccharide phenotypes based strictly on the use of dyes.

Key Words: *Sinorhizobium*, pH, Aniline blue, Congo red, Calcofluor white

Bacteriological dyes have been used as an important component in medium to determine production of various polysaccharides and extracellular elements. In particular, Congo red, Calcofluor white, and Aniline blue have seen prominent use in bacteria, plants, and fungi. Congo red and Calcofluor white are typically used as dyes to stain polysaccharides and cellular elements containing $\beta(1-3)$ or $\beta(1-4)$ linkages, and have particularly strong interactions with cellulose and chitin (Hughes and McCully 1975; Kneen and Larue 1983; Wood 1983; Mori and Bellani 1996; Rivera *et al.* 2012). However, the use of these dyes is not limited to polysaccharides since Congo red can also bind to extracellular fibers called Curli produced by *Escherichia coli* (Reichhardt *et al.* 2016). The use of these dyes has also had an impact on the ability to isolate symbiotically effective rhizobia. Most *Rhizobium* strains lack the ability to bind Congo red when grown on YEM (Zevenhuizen *et al.* 1986). However, non-nodulating strains of *Rhizobium leguminosarum* *bv. trifolii* have been isolated on the basis of binding to Congo red. This has resulted in the use of Congo red as a potential indicator of nodulating strains of *R.leguminosarum* *bv. trifolii* in mixed cultures (Bromfield and Jones 1980). Calcofluor white is used as an indicator for production of the polysaccharide succinoglycan (EPS-I) in *S. meliloti* Rm1021, and it has long been used as a screening tool to isolate mutants which affect production of this symbiotically important polysaccharide (Leigh *et al.* 1985, 1987; Long *et al.* 1988).

The dye Aniline blue is largely utilized to detect the production of callose, a linear $\beta(1-3)$ glucan found in cell walls in both plants and fungi (Stone *et al.* 1984). This is visualized through a bright yellow fluorescent phenotype when observed under UV light. Fluorescence is due to the interaction of a fluorochrome from Aniline blue (Sirofluor) interacting with $\beta(1-3)$ linkages (Evans *et al.* 1984). While bacteria do not produce callose, production of similar polysaccharides consisting of $\beta(1-3)$ linked glucans has been observed, including the polysaccharide curdlan

(Kenyon and Buller 2002; Stone 2005). Production of curdlan is known to occur in various bacteria including some *Agrobacterium* species (Stasinopoulos *et al.* 1999). The production of curdlan is visualized in media supplemented with Aniline blue by cell biomass turning a blue colour (Stasinopoulos *et al.* 1999).

The production of polysaccharides can be heavily influenced by many factors such as pH, salt concentration, osmolarity, and the ability to metabolize carbon sources (Dylan *et al.* 1990; Miller-Williams *et al.* 2006; Hellweg *et al.* 2009; Geddes *et al.* 2014). Previous work to identify *S. meliloti* mutants unable to catabolize galactose led to the isolation of a Tn5 insertion mutant in *dgoK1* (SRmD304), which was observed to acidify medium when grown in the presence of galactose (Geddes and Oresnik 2012a, 2012b; Geddes *et al.* 2014). When SRmD304 was initially streaked on Rhizobium Minimal Medium (RMM) (Broughton *et al.* 1986) using galactose and glycerol as carbon sources, a mucoid phenotype was observed suggesting an increased production of EPS. However, when the strain was streaked onto RMM containing Calcofluor it exhibited a dull fluorescent phenotype that seemed to be atypical for EPS-I (Figure 1c, top panel).

To further investigate the produced polysaccharide, SRmD304 was screened on medium supplemented with either Aniline blue, Congo red, or Calcofluor white. Rm1021 (Wild-type) and SRmD304 were grown at 30°C overnight in 5 mL cultures of LB broth (Sambrook *et al.* 1989) to an OD₆₀₀ of 1.0. A 1 mL aliquot of each culture was pelleted and re-suspended in 100 µL of 0.85% w/v saline. Finally, 20 µL of this cell suspension was spotted onto YMA (Yeast Mannitol Agar) agar (Kneen and Larue 1983) supplemented with 15 mM galactose and Congo red (25 µg/mL), and RMM supplemented with 15 mM galactose and either Aniline blue (50 µg/mL) or Calcofluor white (0.02%).

The results showed that after 5 days at 30°C Rm1021 had a negative reaction on plates supplemented with either Congo red or Aniline blue (Light orange and white respectively) (Figure 1, top panels). However, a Calcofluor bright phenotype was observed suggesting only succinoglycan was being produced, which is consistent with previous results (Zevenhuizen *et al.*, 1986; Leigh and Lee, 1988). SRmD304 gave a positive result with both Aniline blue and Congo red, turning blue and red respectively, while displaying a dull fluorescence under UV on plates containing Calcofluor white. A possible interpretation of this result was that in the presence of galactose SRmD304 may be producing a novel EPS that contained $\beta(1-3)$ linkages; possibly curdlan. The operon *crdASC* encodes enzymes involved in the biosynthesis of curdlan in *Agrobacterium* (Stasinopoulos *et al.* 1999), however orthologues to these genes are not found in *S. meliloti* Rm1021 genome.

Further characterization of SRmD304 showed that, when the *dgoK1* strain was grown in weakly buffered conditions in the presence of galactose and a secondary carbon source that it could metabolize it would acidify its growth medium to pH 4.5, and that the EPS that was produced had a 7:1 glucose:galactose ratio that was indicative of succinoglycan (Geddes *et al.* 2014).

Many dyes are known to be affected by media pH, including Congo red, Aniline blue, and Calcofluor (Darken 1962; Evans *et al.* 1984; Mera and Davies 1984). To determine if the observed dye phenotypes associated with *dgoK1* resulted from medium acidification, the mutant and the wild-type strains were spotted onto plates that were modified by adding 50 mM MOPS buffered at either pH 6 or pH 6.5. The results show that when the wild-type (Rm1021) and the strain carrying the *dgoK1* mutation (SRmD304) were spotted onto plates buffered at pH 6.5 the phenotypes that were noted for SRmD304 on the weakly buffered plates differed. The intensity

of the staining with Congo red and Aniline blue decreased, whereas the fluorescence associated with Calcofluor white increased (Figure 1, middle panels). In contrast, when the strains were spotted onto plates buffered at pH 6 the phenotypes of both the wild-type and the strain carrying the *dgoK1* mutation appeared similar to that seen when SRmD304 was spotted onto the weakly buffered media, resulting in a positive reaction to Aniline blue and Congo red while being Calcofluor dull (Figure 1, bottom panels). Taken together these observations support the hypothesis that the dye phenotypes associated with the *dgoK1* mutation were due to medium acidification.

The influence of pH on Aniline blue phenotypes has not to our knowledge been previously shown at this pH range. It was reasoned that if the positive reaction of Aniline blue is correlated with medium acidification in *S. meliloti*, this property could be exploited to screen for mutant strains unable to acidify their growth medium. To test this hypothesis, SRmD338, which carries an unmarked deletion of *dgok1* (Geddes 2014), was mutagenized with Tn5 as previously described (Finan *et al.* 1988). The resulting Tn5 mutants were screened on RMM supplemented with 15 mM galactose and Aniline blue. Mutants that were phenotypically white were purified, and the site of insertion in the *S. meliloti* genome was determined using arbitrary PCR (Miller-Williams *et al.* 2006). Two such mutants were isolated in this manner; one in the gene *SMc00588*, which has been previously shown to be necessary for medium acidification in a *dgok1* mutant background (Geddes *et al.* 2014), and one in the gene *tkt2*, encoding for a putative transketolase. The *tkt2*::Tn5 mutation was transduced into Rm1021 to further investigate phenotypes associated with this mutation. It was determined that a strain carrying this mutation had a carbon utilization phenotype similar to those reported for transketolase mutants in *E. coli* (Zhao and Winkler 1994). The growth phenotypes were fully complemented by the introduction

of a plasmid over-expressing *tkt2* (Data to be presented elsewhere).

To determine if the Aniline blue phenotype associated with SRmD373 (Δ *dgok1*, *tkt2*::Tn5) was due to an inability to acidify its growth medium, broth cultures of SRmD373 were grown in RMM medium containing both glycerol and galactose as previously described (Geddes 2014). Consistent with what was previously observed, the wild-type (Rm1021) did not acidify its growth medium whereas supernatant of the SRmD338 (Δ *dgok1*) growth medium dropped to a pH around 4.5 (Figure 2). In contrast, SRmD373 (Δ *dgok1*, *tkt2*::Tn5) was markedly impaired in its ability to acidify its growth medium (Figure 2).

The isolation of a mutation in *tkt2* suggests alteration of central carbon metabolism plays a role in the observed galactose dependent medium acidification observed in SRmD338. This could be due to altered carbon flow to compounds which are secreted and subsequently drop the pH of the medium. Medium acidification in the *dgok1* background has been shown to be dependent on galactose dehydrogenase activity (SMc00588), suggesting that galactonate accumulation plays a role in altering the medium (Geddes and Oresnik 2012b). The observation that a mutation in *tkt2* results in a similar phenotype suggests that medium acidification may not be solely dependent upon SMc00588. We have also observed that a strain carrying only a *tkt2* mutation has an Aniline blue negative phenotype (white) under conditions which Rm1021 stains blue (Data to be presented elsewhere). This suggests a mutation in *tkt2* may affect medium acidification independent of the *dgok1* mutation and galactose. Since the loss of transketolase represents a major block in central metabolism, it seems plausible that altered carbon flow to other metabolites may also affect medium acidification. Of note, it has been recently shown that proteins associated with the pentose phosphate pathway are increased when *S. meliloti* is grown in acidic conditions (Draghi *et al.* 2016).

The results observed here show previously un-reported dye phenotypes of Aniline blue, Calcofluor white and Congo red. While it is well known that these dyes have pH components, it is usually at extreme pH ranges, and have not been reported around pH 6 – pH 6.5. Although our results suggest that the attributes associated with Aniline blue can be exploited to screen for phenotypes, it is important to emphasize that these results suggest caution should be used when using these dyes to assign polysaccharide phenotypes if they are being used in weakly buffered media.

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Figure legends

Figure 1. Dye phenotypes in buffered media. Observed dye phenotypes of strains on plates supplemented with **A)** Congo red **B)** Aniline blue, and **C)** Calcofluor white. Bacterial spots were scored after 5 days on media that was buffered as labeled. Calcofluor white images were observed during exposure to UV light.

Figure 2. Measurement of culture pH for each strain over 72 hours. The data presented are an average of 3 independent biological replicates. Where not visible error bars are smaller than the size of data points.

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Figure 2

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