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Curcumin as an Amyloid-indicator Dye in *E. coli* †

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Abstract

We have demonstrated that curcumin is an amyloid-specific dye in *E. coli*. Curcumin binds to curliated whole cells and to isolated curli amyloid fibers. Similar to Congo red, curcumin exhibits a red-shift in absorbance and a significant increase in fluorescence upon binding to isolated curli.

Since the late 1800s, staining of cells and cell structures by small molecule dyes has proven to be an indispensible tool in microbiology[1]. Today, dyes are crucial tools in the study of bacterial communities called biofilms. Biofilms consist of bacterial cells plus their secreted extracellular matrix comprising a complex mixture of proteins, polysaccharides, and sometimes DNA[2]. Due to their chemical and structural complexity and insolubility, biofilms are difficult to analyze. However, indicator dyes such as crystal violet, Calcofluor White, and Congo red enable rapid screening of biofilm production and qualitative identification of biofilm components[3–5].

The anionic diazo dye, Congo red (CR), is well known for its amyloid binding properties[6]. Indeed, the definitive diagnosis of Alzheimer's disease is made upon post-mortum CR staining of brain tissue[7]. The use of CR as an indicator dye also has a rich history of applications in microbiology and has been used extensively to detect the production of various extracellular polysaccharides and proteins in bacteria and in yeast[8–13]. In the case of *E. coli* and *Salmonella*, these organisms produce extracellular fibers termed curli[14, 15], involved in adhesion and biofilm formation, that were discovered to be amyloid in 2002[16]. Since the discovery of curli as amyloid, an increasing number of microbial functional amyloids have been identified and are being investigated to understand their function in microbial ecology and to dissect amyloid-assembly processes[17].

CR has been routinely employed to score the production of curli among *E. coli* and *Salmonella* species[18, 19]. CR-binding phenotypes have been valuable in studies of curli biogenesis in *E. coli* strain MC4100, a strain frequently used to manipulate curli assembly[16]. When grown on CR-containing agar medium, curliated whole cells bind CR and deplete the dye from the underlying agar. However, because CR can bind to other cellular features in some strains, the curli-dependence of dye binding must first be established.

Here, we demonstrate that CR binding does not exhibit curli specificity in the biofilm-forming uropathogenic *E. coli* strain UTI89, a well-studied strain in models of urinary tract bacterial pathogenesis. We also report our discovery that the natural product curcumin binds *E. coli* cells in a curli-dependent manner. We grew two strains, MC4100 and UTI89 along with their respective curli knock-out strains, MC4100Δ*csgA* and UTI89Δ*csgA*, on YESCA agar supplemented with 25 μg/mL CR for 60 hours at 26 °C, typical growth conditions that promote curli expression[13]. MC4100 does not form biofilms as MC4100 can produce curli

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but does not produce cellulose, a determinant of biofilm formation in *E. coli*. UTI89 produces both curli and cellulose which contribute to its ability to form biofilms on agar, on plastic, and at the air-liquid interface[20]. As expected, CR binds to the curli-producing strains MC4100 and UTI89 and does not bind to MC4100Δ*csgA*, a curli mutant lacking the major fiber subunit protein (Figure 1). However, the UTI89 curli mutant, UTI89Δ*csgA*, binds CR, although it does not produce curli (Figure 1). Each strain was also grown in the absence of CR on standard YESCA agar and then harvested, resuspended in PBS, and normalized to yield an OD_{600} 1.0 suspension in 1 mL PBS.

CR from a concentrated stock solution was added to each sample to yield a final concentration of 10 μg/mL CR. Cells were incubated for ten minutes in a microcentrifuge tube, centrifuged at 9600 g, and photographed. As illustrated in Figure 1, CR is also able to bind to both UTI89 and UTI89Δ*csgA* whole cells in this assay. Both assays illustrate the lack of specificity and problems associated with using CR staining as an amyloid dye for many microorganisms. Indeed, CR is known to bind to cellulose[21, 22], a common biofilm component. UTI89 produces both curli and cellulose in the extracellular matrix, whereas MC4100 produces only curli. Thus, CR binding is curli-specific in MC4100 because it does not also produce cellulose. The presence of cellulose is often identified qualitatively through Calcofluor binding and fluorescence. As shown in Figure 1, both UTI89 and UTI89Δ*csgA* exhibit Calcofluor-based fluorescence when grown on a YESCA agar plate containing 20 μg/mL Calcofluor. MC4100 and MC4100Δ*csgA*, which do not produce cellulose, do not exhibit significant Calcofluor-based fluorescence. Interestingly, for the UTI89 pair, the fluorescence of Calcofluor is reproducibly somewhat greater in the absence of curli. It is possible that Calcofluor binding to cellulose is inhibited by curli-cellulose interactions or that the environment in the presence of curli leads to reduced fluorescence.

Naturally, more specific amyloid dyes as alternatives to CR would be valuable for use in microbiological assays. We discovered that the natural product curcumin, also studied for its ability to interact with amyloid β [23], was amyloid-specific and served as a reliable indicator of curli production in UTI89 (Figure 1). Curucmin uptake is obvious among MC4100 and UTI89 grown on YESCA agar medium containing 18 μg/mL curcumin, whereas MC4100Δ*csgA* and UTI89Δ*csgA* do not bind curcumin. It is conceivable that the lack of curcumin binding on agar could be attributed to possible inhibition of cellulose production during growth in the presence of curcumin. Thus, the solution-based pull-down assay using cells grown in the absence of curcumin is important to confirm curli-binding specificity. The assay was also performed using Calcofluor. The molecule concentrations were 0.1 μg/mL Calcofluor and 1.8 μg/mL curcumin. The results of these pull-down assays paralleled what was observed in the cells grown in compound-supplemented agar. Notably, UTI89 binds curcumin in the pull-down assay and appears yellow, whereas UTI89Δ*csgA* does not and appears white. Thus, curcumin binding is curli specific, even in the presence of cellulose and any other extracellular components in UTI89Δ*csgA*.

CR has also been examined in many biophysical studies of amyloid fibers *in vitro*, and is noted for its red-shift in absorbance as well as its characteristic fluorescence when bound to amyloid fibers [24]. Thus, we examined the ability of curcumin to interact with curli fibers *in vitro* and whether curli-bound curcumin would exhibit similar properties. CR or curcumin were individually added from a 0.5 mM stock solution in DMSO to a solution of 1 mg/mL of isolated curli, to yield a final dye concentration of 25 μM. Each sample was examined by UV-Vis spectrophotometry. The fluorescence emission spectra of each dye alone and in the curli-bound samples were also obtained upon excitation at 540 nm for CR and at 432 nm for curcumin. As shown in Figure 2, the UV- Vis spectrum of curli-bound curcumin is red-shifted from a λ_{max} of 423 nm to 432 nm. Furthermore, although curcumin itself exhibits some detectable fluorescence, there is a significant increase in the fluorescence emission,

centered at 521 nm, when bound to curli, similar to the behavior of Congo red (Figure 2). In addition, the fluorescence intensity is greater for curcumin than for CR. Thus, curcumin also exhibits the useful spectral properties ascribed to CR when bound to isolated curli[16].

Since the discovery of curli as amyloid in 2002, there is an increasing number of functional amyloids being identified among microbial species and the identification of amyloid-specific dyes for use in the chemically complex background of microbial biofilm communities will be of value. We have demonstrated that curcumin is an amyloid-specific dye in *E. coli* and may prove to be valuable in examining amyloid production among other environmental and host-associated microbial species.

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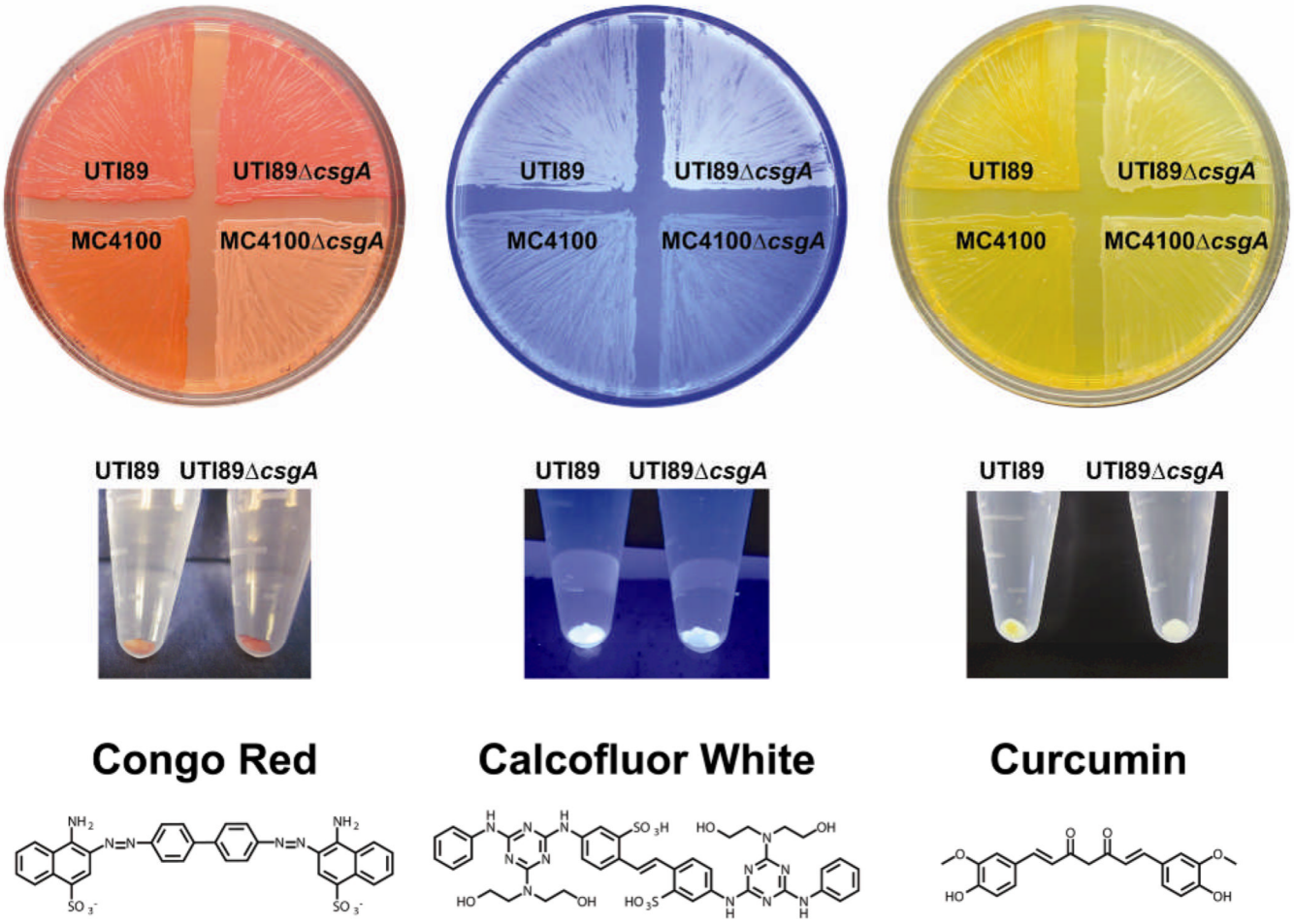

Figure 1.

Dye binding to MC4100 and UTI89 wild-type and curli-mutant strains during growth on YESCA agar plates supplemented with the indicated molecules (top row) and after growth on YESCA agar without compound supplementation via a solution-based pull-down assay (middle row). CR binding is not curli specific in UTI89. Calcofluor-based fluorescence indicates the production of cellulose by UTI89 and UTI89Δ*csgA*. Curcumin exhibits curli-specific binding in UTI89 even in the presence of cellulose.

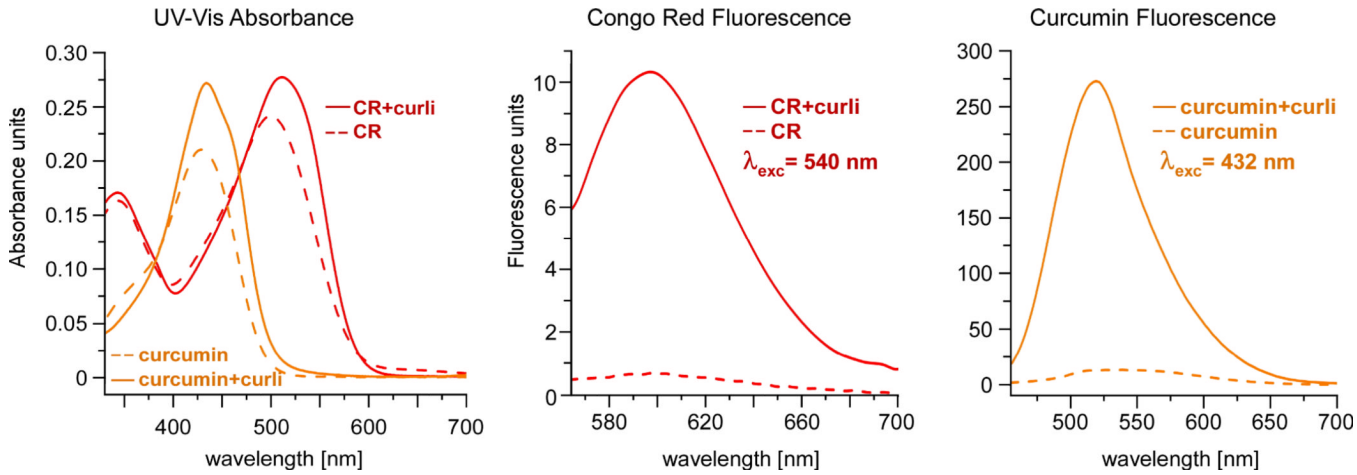

Figure 2.

Binding of curcumin to curli results in a red shift in the absorbance spectrum, similar to the spectral shift observed for Congo red and commonly used to characterize amyloids (left). Like Congo red, curcumin exhibits significantly enhanced fluorescence when bound to curli (middle and right).