Artificial Fluorogenic Substrates in Microfluidic Devices for Bacterial Diagnostics in Biotechnology

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Providing new fluorogenic substrates with designed enzyme-labile moieties for microfluidic live cell analysis is an innovative complementary approach to conventional cultivation based methods of bacterial diagnostics. The advance of their integrated application in microfluidic devices is presented in comparison to established approaches. A comprehensive insight on recent implementation is given and highlighted with a commercially available example.

Keywords: fluorogenic substrate, coumarin, calcein acetoxymethyl ester, enzyme-labile moieties, bacterial diagnostics, clinical diagnostics

1. Introduction

Fluorogenic substrates are nonfluorescent due to quenching moieties until those are cleaved off enzymatically. Those artificial substrates can be generated by conventional click chemistry [1] or are commercially provided in a broad range. Fluorogenic substrates are enzymatically converted to fluorescent products and can be designed according to distinguishing metabolic features of pathogens or bacterial classes. Appearance of fluorescence reveals information about uptake, conversion, and efflux of those chemicals and their products.

Their innovative implementation in microfluidic analytics of microorganisms was demonstrated recently [2, 3]. Novel applications with those chemical compounds for bacterial detection [4–6], metabolic characterization [2, 7, 8], drug development [9], preclinical studies [10], and screening for antibiotic resistance [11] are reported.

Important scaffolds are from the fluorochrome family of xanthene dyes as fluorescein and rhodamine derivatives or the family of coumarins as 4-methylumbelliferone, resorufin, and 7-hydroxycoumarin as reviewed elsewhere [12] and also other fluorescent scaffolds as, e.g., oxy-luciferin [10] or artificial fluorochromes as Cy3 or Cy5.5 [11].

The application of commercially available fluorogenic or chlorogenic substrates and their conversion in conventional bacterial diagnostics is typically performed by time-consuming and cumbersome media or gel-based assays. These methods enable detection and characterization of microorganisms by their characteristic enzyme activities only after days of incubation and if colony growth is given [13]. Unfortunately, not all bacteria of relevance are in a culturable state.

Selective media for microbiological diagnostics relying on biocatalytical conversion are described in literature, and they are commercially available for a long time [14]. Unfortunately, extended cultivation and incubation times often lead to long delay times of days up to weeks until data evaluation can be performed [15]. The results of agar plate count methods are typically a whether or not finding, depending on a positive or negative conversion of the selective media components after days of incubation. Therefore, for every sample, many agar plates have to be prepared, plated, and analyzed one after another due to mandatory serial dilutions to generate separated superficial colony forming units of bacterial suspensions with unknown cell number (Figure 1A).

Usually, pharmaceutical and clinical diagnostics depend on reliable high-throughput measurements for fast and precise analysis of high sample loads. For this purpose, fluorescence measurements with the automated plate readers [16] or flow cytometry (FCM) [17] have improved sample throughput (Figure 1B and 1D). Further, microtiter well plates facilitate parallelization and automation of sample analysis by the use of plate readers and robotic liquid handling stations. The fluorogenic substrate is provided in the liquid medium of the cavities, and fluorescence measurements can be performed dynamically with different substrate concentrations or as endpoint analysis of several bacteria isolates.

FCM allows snapshot analysis of single bacterial cells and their viability or cell functionality [15, 18]. However, bacteria have to be cultivated and sampled, and intracellular fluorescence has to be generated by genetic modification for introduction of fluorophore expression, by labeling with fluorescent dyes or previous cultivation in shaking cultures with fluorogenic substrates prior analysis. Nevertheless, FCM still requires offline sample preparation, and storage before results can be acquired. Obviously, sample preparation and delay in analysis can influence measurements especially in a dynamic enzymatic reaction system prevailing in living bacteria. Although the analysis mode is fast and automated, samples have to be analyzed successively and temporal resolution is very limited. A great advantage of FCM is the analysis of heterogeneities in bacterial populations.

These single-cell resolved analyses can also be performed in microfluidic cultivation devices (Figure 1C), which integrate cultivation and spatiotemporal analyses. Further, microfluidic devices offer the opportunities of volume reduction, reduction of expensive reactions reagents, parallelization of sample measurements, and flexibility in device design. The trapping of single cells is sufficient without the requirement of high cell numbers as it is the case for plate count methods, plate reader based assays, FCM analysis, or gel-based diagnostics.

Therefore, we would like to draw the attention on recent advances on synthesis and implementation of fluorogenic substrates for analysis of native bacterial enzymes of crude extracts or living cells. The importance of nontoxic fluorogenic compounds is shown with a commercially available calcein acetoxymethyl ester derivative.

1.1. Relevance of Novel Nontoxic Fluorogenic Substrates and Recent Approaches of Their Application. The synthesis of fluorogenic substrates and their application for conversion by specific enzymes of pathogens and nonpathogenic bacteria is of interest to detect bacteria prior to drug therapy of patients [5], to define pathogenicity [7], or to find possible drug targets to tackle bacterial resistance by screening of lead component libraries of production strains [1]. We summarized recent analytical approaches using fluorogenic substrates in Table 1 that have the potential to be advanced to microfluidic bacterial diagnostics methods or already using dynamic analysis systems.

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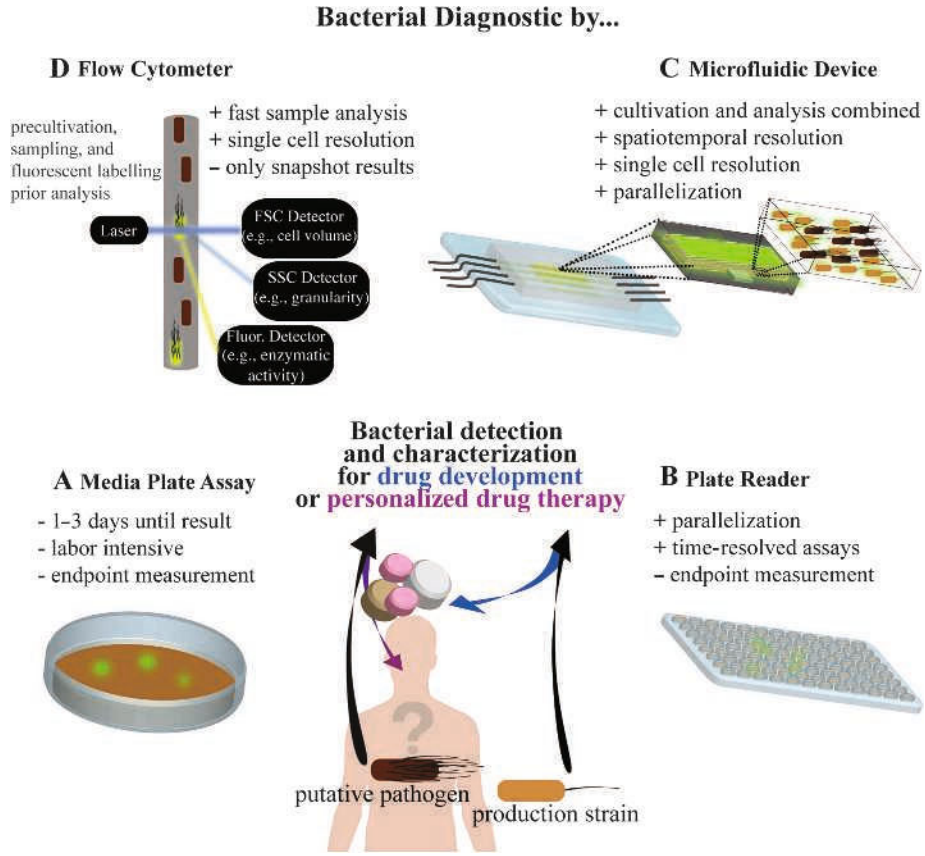

Figure 1. Fast, reliable, and high-throughput bacterial detection for putative pathogens and characterization of production strains is of high relevance for clinical diagnostic, pharmaceutical drug development, and biotechnological production. (A) Conventional bacterial diagnostics utilizing fluorogenic substrates is still mostly a two-step approach separating conventional plate cultivation and subsequent analysis. (B) Miniaturization and parallelization are realized in multiwell plate assays without single-cell resolution. (C) Microfluidic cultivation with a continuous supply of fluorogenic substrates can integrate and parallelize sample analyses. In combination with time-lapse microscopy, microfluidic cultivation approaches facilitate spatial and temporal resolution of bioconversion at the single-cell level. Thus, comparable low cell numbers and short cultivation phases suffice for bacterial analyses. (D) Flow cytometry facilitates fast single-cell snap shot analysis at tremendous throughput. However, conventional precultivation and sample preparation steps are necessary

Thus, microfluidic approaches will gain importance in the future due to their advances of sample analyses with spatiotemporal resolution and potential of sample free measurement automation with lowered reagent usage. However, prior synthesis of fluorogenic compounds with enzyme-labile moieties is required before bacterial diagnostics of enzymatic activities can be established. A recent application of previously synthesized artificial β-lactamase substrates containing green fluorescent Tokyo Green has

Table 1. Selection of fluorogenic substrates and their use in bacterial diagnostics in static and perfused analysis systems

	Fluorogenic substrate	Enzyme activity	Analysis system	Reference
Static analysis systems	7-Hydroxy-3 h-phenoxazin-3-one 10-odxide (resazurin)	Metabolic activity of periodontopathic bacteria	Spectrophotometer	Ishiguro et al., 2015 [4]
	4-Methylumbelliferyl butyrate, 4-Methylumpelliferyl heptanoate, 4-Methylumbelliferyl oleate	Cell wall-anchored carboxylesterase (*M. tuberculosis*)	Well plate reader	Lun and Bishai, 2007 [7]
	N-Acetyl tripeptide-aminomethylcoumarins	Protease (*Mn tuberculosis*)	Well plate reader	Akopian et al., 2015 [9]
	5-Nitrofuryl caged luciferin	Bacterial nitroreductases	Well plate reader and preclinical in vivo imaging of mice	Vorobyeva et al., 2015 [10]
	7-Hydroxy-9 h-(1,3-dichloro-9,9-dimethylacridin-2-one-acetoxymethylesterresorufin-acetoxymethylester	Mycobacterial esterases	Native PAGE	Tallman and Beatty, 2015 [8]
Dynamic analysis systems	Carboxyfluorescein diacetate and carboxyfluorescein diacetate succinimidyl ester	Bacterial esterases	Flow cytometry	Hoefel et al., 2003 [5]
	Fluorescein diphosphate	Alkaline phosphatase	Time-lapse imaging	Shim et al., 2009 [2]
	LRBL1-3 (β-lactamase-responsive bacterial labelling)	β-Lactamase	FRET-based live cell imaging and flow cytometry	Shao et al., 2013 [11]
	β-D-cellobioside-6,8-difluoro-7-hydroxycoumarin-4-methanesulfonate	Cellobiohydrolase	Microfluidic FCM	Najah et al., 2014 [19]
	CDG-1 and CDG-OMe	β-Lactamase	Microfluidic FCM	Lyu et al., 2015 [6]
	Fluorescein dicapylate	lipases	Time-lapse imaging	Hosokawa et al., 2015 [20]
	Cyto Calcein 450 acetoxymethyl ester	Bacterial esterases	Time-lapse imaging	Krämer et al., 2015 [3]

been presented for *Mycobacterium tuberculosis* detection in microfluidic droplets [6].

Smart substrate design enables diagnostic concepts with coupled enzymatic reactions as shown for a caged luciferin that requires conversion by nitroreductases prior reaction with luciferase for metabolic characterization of living bacteria [10]. Furthermore, enzymatic cleavage of quencher moieties can be used for analytical FRET biosensor construction [11].

In addition, enzyme activity screening for fine chemical production bears a high motivation to synthesize tailored fluorogenic substrates as demonstrated by microfluidic microdroplet biotransformation of bacterial libraries with cellolytic and lipolytic enzyme activities [19, 20]. These hydrolyases are of relevance for fine chemical production or biofuel production [19, 20].

Although studies with fluorogenic substrates are still performed nowadays partly in static analysis systems without single-cell resolved results, they have a high potential to be advanced in combination with microfluidic devices for microbial cultivation [21].

The future potential of these microfluidic devices for in-flow bioanalysis can be demonstrated with calcein acetoxymethyl esters (CAMs), which are used conventionally for noninvasive mammalian cell analysis [22, 23]. CAMs are quenched by covalently ester groups belonging to the fluorochrome family of xanthene and coumarin derivatives, respectively. After cellular uptake, the ester groups are cleaved off the fluorescent calcein followed by equimolar ethanol formation [23].

1.2. Conversion of a Calcein Acetoxymethyl Ester for In Vivo Single-Cell Analysis. A disadvantage of conventional bacterial diagnostic methods is that nongrowing bacteria are easily overseen. Dormancy of bacteria is characterized as a nonreplicating state with reduced enzyme activities. Thus, methods relying on enzymatic activity have to be very sensitive towards low cell numbers and decreased substrate conversion.

Notably, dormant bacteria are of relevance in pharmaceutical research and clinical treatment because they bear an increased potential for antibiotic tolerance, hidden infections, and bacterial persistence. Usually, nonsporulating *Actinobacteria* are reported to develop dormant phenotypes that can exist as latent (dormant) infections in patients without symptoms of the disease, endure medication, become drug resistant, and initiate acute infections in patients [24]. Gengenbacher and Kaufmann (2012) summarized the important facts of dormancy related to *M. tuberculosis* that is one of the most severe infection diseases of the world [24].

Commercially available CAM is highlighted here for bacterial diagnostics to give a perspective for future microfluidic approaches of microbiological assay performance. Experimental procedures are described in detail in Krämer et al. (2015) [3]. CAM concentrations from 23.1 μM to 138.9 μM were dissolved in the perfusion medium of the microfluidic cultivation device, to test if increasing CAM concentrations influence the cell growth of the organism *Corynebacterium glutamicum*. The microorganism is not only relevant for fine chemical production, but it is also a nonpathogenic relative of the human pathogen *M. tuberculosis* and other human diseases.

The nontoxic, intracellular conversion of commercially available Cyto Calcein 450 by growing bacterial microcolonies of *C. glutamicum* is indicated by the exponentially increasing colony fluorescence within the first 5 h (Figure 2A). During the experiment, intermediate energy driven efflux of the well-retained fluorochrome calcein was found to be inhibited by cultivation in the absence of glucose. A lack of carbon caused increasing mean single-cell fluorescence, whereas the mean colony fluorescence

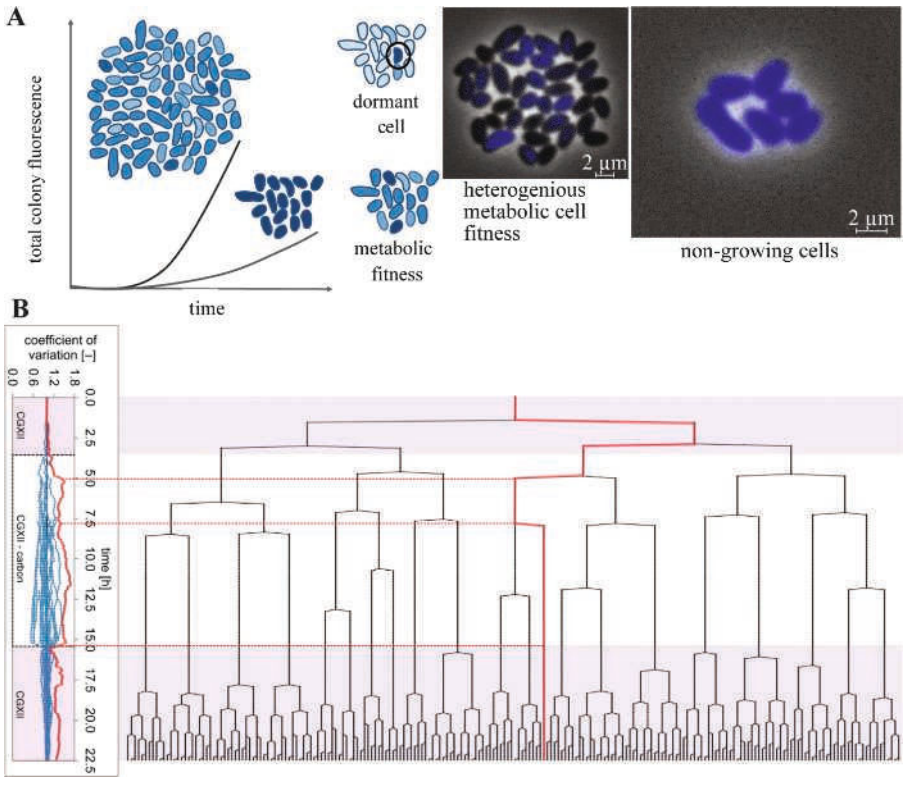

Figure 2. Fluorogenic substrate conversions for microbial single-cell analysis combined with microfluidic cultivation and time-lapse imaging. (A) Apparent conversion of calcein acetoxymethyl ester by a microbial colony increases exponentially with growing cell numbers. Heterogeneity of intracellular fluorescence of single cells can be observed due to fluctuations of metabolic activity of individuals or as reaction to environmental conditions (as shown in the micrographs). (B) Exemplary microcolony lineage of *C. glutamicum* grown on continuous supply of CGXII medium with an intermediate famine phase (3 h–15 h) with medium lacking carbon source. Despite the artificially introduced growth arrest by nutrient deprivation, real-time mean single-cell fluorescence provided information about metabolic activity of every single cell and heterogeneity in the colony. Reduction of metabolic activity due to a dormant state decreases calcein efflux. Dormant cells (highlighted in red) were detected by an increased coefficient of variation of their mean intracellular fluorescence in comparison to the mean fluorescence of all siblings in the colony

remained constant due to stagnation of total cell number. Usually, dormant cells, which decrease their metabolic activity and have high relevance in pharmaceutical and clinical diagnostics, can be distinguished due to significantly increased single-cell fluorescence in comparison to the average (Figure 2B).

Notably, the apparent conversion of CAM by the growing *C. glutamicum* colony depended on the increasing total cell number (Figure 3A–3B). According to differences in cell fitness, cell size, and cell cycle, individual variations in intracellular fluorescence increased especially during intermediate starvation stress without carbon feed and afterwards. The slope of the mean colony fluorescence due to exponential cell growth could be used to qualify cultivation conditions in comparison to the reference condition in complex medium BHI at pH 7.0. The apparent mean single-cell reaction rate constant has been determined for apparent CAM conversion from the slope of the mean colony fluorescence over time. This allows evaluating the physiologic impact of different cultivation conditions and media (Figure 3C–D). Results can be exploited for drug-development, preclinical analyses, and personalized medicine to treat infections with the appropriate drug in efficient concentration. The impact of a short (1 h) perfusion of bacterial colonies with 10 µg/mL chloramphenicol (CHL) or ampicillin (AMP) demonstrates that the microfluidic approach gives results after several hours instead of waiting 1–3 days, revealing insight about remaining metabolically active bacteria, and can be used for drug therapy optimization in future.

2. Conclusion and Outlook

The interdisciplinary approach of chemical design of new fluorogenic substrate moieties, their bioanalytical application, and engineering microfluidic cultivation devices has a broad perspective for microbiological characterization, biocatalytic activity screening, and bacterial diagnostics by enzymatic reactions. The possibilities of analytical flow chemistry are not fully exploited by far for bacterial diagnostics especially in low cell numbers. However, enzyme activities of single mammalian cells have already been discovered with continuous or intermittent fluorogenic substrate feed in microfluidic cultivation devices [22, 23]. A broad innovative use of fluorogenic substrates lies in approaches for rapid future bacterial diagnostics or for rapid enzyme activity screening of bacterial libraries in hours instead of days with the possibility of cell sorting integrated in microfluidic devices [20].

Chemical synthesis of organic molecules appropriate for bacterial uptake and relevant enzymatic activity specificity has a great future perspective for in-flow biochemistry. The conversion of fluorogenic compounds during cultivation answers if bacteria are viable under the condition of interest [3, 4, 10]. The role of enzymes in virulence and bacterial survival is of high interest for basic research as well as future oriented therapeutic strategies [7, 9, 11]. Enzyme expression in expression hosts bear the risk of accumulation of inclusion bodies and enzyme denaturation [7]. Furthermore, the expression of cell wall anchored

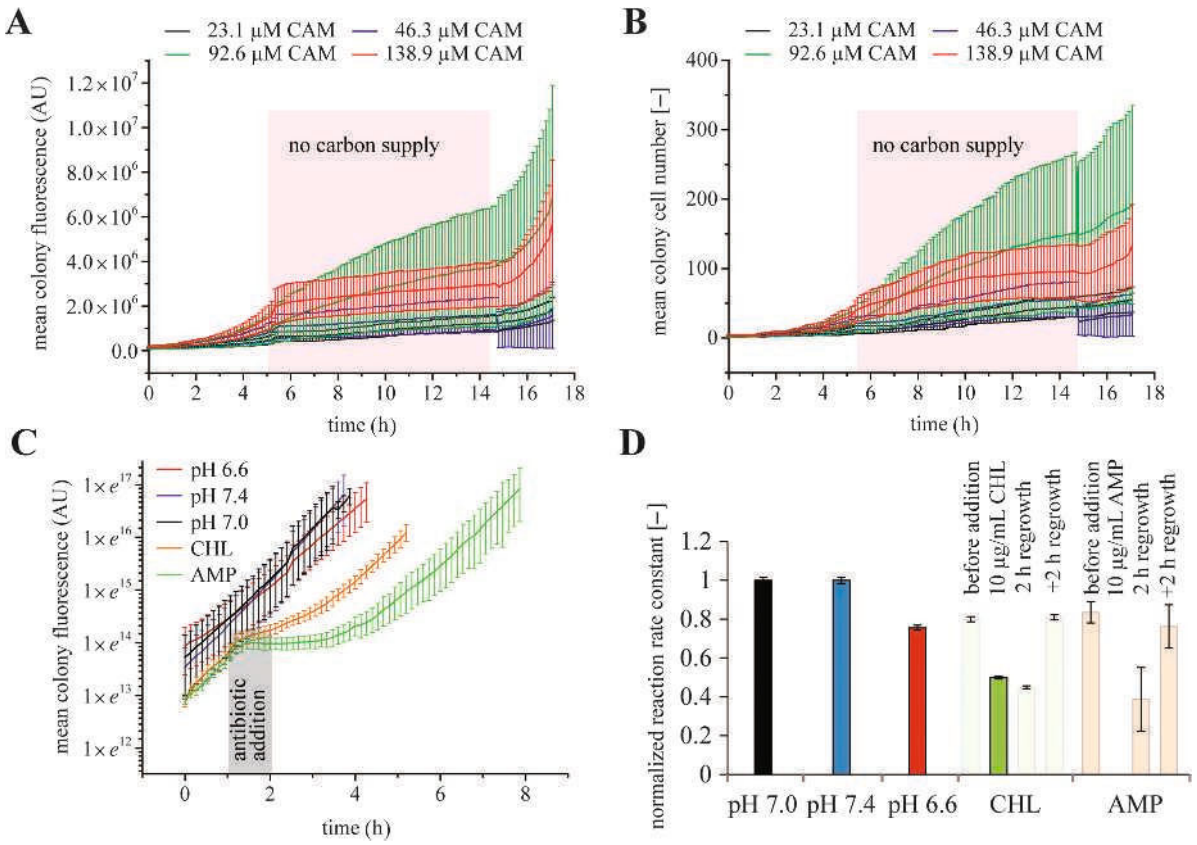

Figure 3. *C. glutamicum* was cultivated in a microfluidic cultivation device with microarrays of picoliter-sized cultivation chambers. The bacteria were supplied with nutrients and dissolved CAM by medium perfusion as described previously [3, 21]. (A) Apparent total colony fluorescence of bacterial colonies fed with 23.1 µM CAM (black lines), 46.3 µM CAM (violet lines), 92.6 µM CAM (green lines), or 138.0 µM CAM (red lines) supplemented to the perfusion medium (CGXII+4% glucose (w/v)). The intermediate phase of glucose free cultivation condition is indicated. For every CAM concentration, five colonies were analyzed to determine if an increase of CAM concentration reduces the mean colony cell number. (B) The corresponding mean total cell number of diagram A is shown over time. (C) Apparent mean fluorescence of five *C. glutamicum* colonies cultivated with complex medium at different conditions. The reference cultivation condition was BHI medium at pH 7.0 without antibiotic addition (black line). Other conditions were pH 7.4 (blue line), pH 6.6 (red line), and with addition of 10 mg/mL chloramphenicol (green line) or 10 mg/mL ampicillin (orange line) for 1 h (as indicated), respectively. (D) The apparent reaction rate constant, derived from the slope of the mean colony fluorescence, was normalized to the reference conditions to validate the impact of stress conditions on the metabolic activity of the model organism *C. glutamicum* given in panel C

enzymes and their purification is quite sophisticated and can be circumvented by analysis of the unmodified microorganism in controlled microfluidic environments.

The design and use of fluorogenic compounds with fluorescent scaffolds emitting red or blue to violet fluorescence [3, 8] opens up the combined use of GFP expression after genetic modification of bacteria [25]. This would give the possibility to determine protein interaction in vivo by multiplexing fluorescence measurements. Therefore, a variation of novel nontoxic fluorescent scaffolds with enzyme-labile moieties will be of rising interest in the future for bacterial diagnostics. Therefore, a nontoxic fluorescent scaffolds with emission variety with minor Stokes shift will be of rising interest in the future. Microfluidic approaches will facilitate screening and diagnostically use new potential fluorogenic substrates to replace conventional incubation until colony growth is given [26].

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