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Dual Strain-Promoted Alkyne–Nitrone Cycloadditions for Simultaneous Labeling of Bacterial Peptidoglycans

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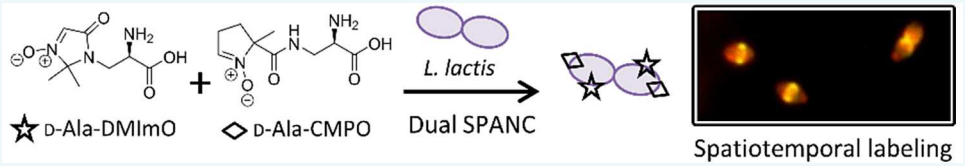
Supporting Information



ABSTRACT: Bioorthogonal chemistry has been applied to study a multitude of biological processes in complex environments through incorporation and detection of small functional groups. However, few reactions are known to be compatible with each other to allow for studies of more than one biomolecule simultaneously. Here we describe a dual labeling method wherein two stereoelectronically contrasting nitrone tags are incorporated into bacteria peptidoglycan and detected via strain-promoted alkyne–nitrone cycloaddition (SPANC) simultaneously. Furthermore, we show orthogonality with the azide functionality broadening the potential for simultaneous biomolecular target labeling in less accommodating metabolic pathways. We also demonstrate the simultaneous labeling of two different food-associated bacteria, *L. innocua* (a model for the food-born pathogen *L. monocytogenes*) and *L. lactis* (a fermentation bacterium). The ability to monitor multiple processes and even multiple organisms concurrently through nitrone/nitrone or nitrone/azide incorporation strengthens the current bioorthogonal toolbox and gives rise to robust duplex labeling of organisms to potentiate the studies of rapid biological phenomena.

INTRODUCTION

Bioorthogonal chemistry, which allows for selective labeling of biomolecules in natural environments, has enabled new opportunities of biological study that were previously inaccessible.[1] This two-step process typically involves metabolic or enzymatic incorporation of a bioinert functional group, followed by subsequent detection through covalent attachment of a reporter tag bearing the paired functional group.[2] One of the most well-characterized bioorthogonal reactions is the copper-catalyzed azide–alkyne cycloaddition (CuAAC).[3,4] However, the toxic effects of the copper catalyst have limited its use in living systems.[5,6] There have been many strides to circumvent use of toxic catalysts,[7−16] broadening the number of bioorthogonal labeling tools available to date; however, limitations still remain, and many are incompatible for detection of multiple biomolecule targets at the same time.

Toward multicomponent biomolecule labeling, new bioorthogonal reactions and combinations thereof are being pursued for the study of more than one target at the same time.[17−24] To date, development of concurrent labeling has mainly focused on uncovering reactions that are mutually orthogonal with each other. The tetrazine ligation has been demonstrated to be compatible with azide, alkyne, or phosphine functionalities in vitro and in living cells, for example.[18−21,23] However, combinations of different reactions can increase systematic error due to potential side reactions or variations in reagent stabilities.[13] Dual labeling with only nitrones and strained alkynes via the strain-promoted alkyne nitrone cycloaddition (SPANC) has potential to avoid such limitations, due to the unique tunability and increased stability of the endocyclic nitrone group (Scheme 1).[25,26]

Stereoelectronically tuned alkyne–nitrone pairs display potential for promising selectivity while maintaining reactivity,[25] which suggests similar pairs could allow for detection of multiple biomolecular targets. We have recently demonstrated that different stable endocyclic nitrone groups can be metabolically incorporated into newly synthesized bacterial peptidoglycan (PG) through functionalization of D-amino acids (Scheme 1C). PG essentially determines the bacterial shape and resistance to cell lysis, and its biosynthetic pathway is a key target in antibiotic development. Since both electronically contrasting nitrones can be metabolically incorporated into PG, we sought to develop a dual bioorthogonal labeling strategy to concurrently distinguish between two incorporated tags in biological systems, as well as provide a new tool for studying peptidoglycan dynamics.

RESULTS AND DISCUSSION

To explore the potential for simultaneous detection of electronically contrasting nitrones incorporated into bacterial

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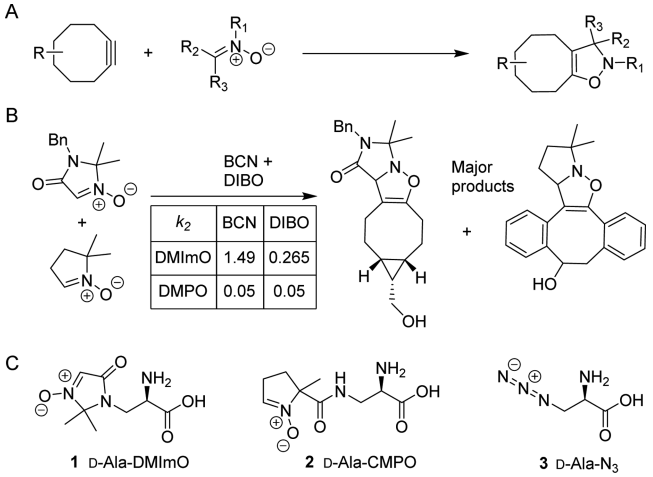
Scheme 1. Stereoelectronically Tuned Alkyne–Nitrone Reactions in SPANC[a]



k_2	BCN	DIBO
DMImO	1.49	0.265
DMPO	0.05	0.05

[a](A) General scheme for SPANC reactions. (B) Major products derived from a one-pot competition from ref 25. Second-order rate constants (k_2, $M^{-1}s^{-1}$) are shown for reactions of nitrones in methanol at 25 ± 0.1 °C with BCN (ref 25) and DIBO (Supporting Information). (C) Unnatural amino acids used in this study.

PG, we compared labeling via SPANC in morphologically different strains of bacteria. Nitrone-bearing D-alanine probes (Scheme 1) were synthesized as previously described[27] and incubated with Gram-positive strains, *Lactococcus lactis* and *Listeria innocua*, as well as with Gram-negative *Escherichia coli*. Excess probe was removed by repeated washes in PBS, and incorporated D-Ala-nitrones, as well as control D-Ala-N_3, were then detected after a portion of each live culture was treated with either CF488-tagged bicyclononyne (BCN) or TAMRA-tagged dibenzocyclooctyne (DBCO) and visualized by fluorescence microscopy (Figures S1–S3).

For *L. innocua* (Figure S1) and *L. lactis* (Figure S2), both electron-poor nitrone **1** and electron-rich nitrone **2** were detected by reaction with DBCO-TAMRA, while BCN-CF488 strictly revealed incorporation of **1**. On the basis of a one-pot competition experiment with the same alkyne–nitrone pairs,[25] it was expected that DBCO would react with both **1** and **2** to a similar degree, and this is reflected in Figures S1 and S2. BCN, however, was expected to react primarily with **1** over **2**, with potential for some labeling of **2**, due to the difference in second order rate constants (1.49 M^{-1} s^{-1} vs 0.05 M^{-1} s^{-1}, respectively).[25] Exploiting this difference in reaction rates and employing short SPANC incubation time appear to have eliminated any low labeling of **2** by BCN. This indicates that incorporated **2** should be easily distinguished from **1** in dual labeling experiments.

While labeling by D-Ala-nitrones appeared to target bacterial cell walls, they had varying fluorescence intensities compared to D-Ala-N_3. Therefore, we wanted to determine if the UAAs followed similar metabolic pathways as natural D-alanine.[28,29] Previously, the Bertozzi group established that bacterial labeling by alkyne[29] and BCN-functionalized[28] D-alanine can be competed for by excess D-alanine. In a similar experiment with *L. innocua*, we found that 10-fold excess D-alanine reduced labeling of **1**, **2**, and **3** all to background fluorescence levels (Figure S4), suggesting access to similar pathways as D-alanine. Although D-alanine can be incorporated into teichoic acids or proteins after racemization to L-alanine, there has been no evidence supporting these routes of incorporation of functionalized D-amino acids based on extensive analyses published to date.[28−30]

To establish dual SPANC as a method for labeling two biological tags simultaneously, we mixed morphologically contrasting *L. innocua* and *L. lactis* to aid in distinguishing between incorporated tags after cycloaddition. Interestingly, both bacterial strains are commonly associated with food, where *L. lactis* is beneficial and involved in cheese production, *L. innocua* is closely related to the food-borne pathogen *Listeria monocytogenes*, which can contaminate unpasteurized dairy products. Separately, the ovococci *L. lactis* were incubated in the presence of **2**, and the rod-shaped *L. innocua* were incubated in the presence of **1**. The cultures were then mixed, washed in PBS, then treated with DBCO-TAMRA, BCN-CF488, or a mixture of both reactive dyes (Figures 1, S5, and S6). Since BCN-CF488 detects only the DMImO tag, while DBCO-TAMRA detects both incorporated nitrones, the merged image clearly allows for discrimination between the two where *L. innocua* labeled with **1** become yellow and *L. lactis* labeled with **2** remain red.

When strained alkyne reactivity was evaluated for the different UAAs, we noted that for *L. innocua* labeled by **3**, there was minimal reactivity with BCN-CF488 which suggests an interchangeable role between azide and CMPO (Figure S1). The second-order rate constant for strain-promoted alkyne–azide cycloaddition (SPAAC) of BCN and benzyl azide was previously measured to be 0.04 M^{-1} s^{-1}, which is significantly lower than the second-order rate constant of BCN in SPANC with DMImO (1.49 M^{-1} s^{-1}).[25] Thus, we hypothesized that SPAAC and SPANC reactions could be mutually orthogonal in a dual labeling strategy. To test this, we used the model system as described above but instead incubated *L. innocua* with **3** and *L. lactis* with **1**. Initial results using equal amounts of UAA indicated that, while being able to positively distinguish between the two strains via dual labeling, the differences in UAA incorporation made it difficult to image labeling patterns for both strains without signal saturation of *L. lactis* (Figure S7a). This was remedied by simply incubating *L. lactis* with one-tenth of the concentration of **1** to bring signal intensities closer together and allow for visualization of what appears to be active sites of cell division, as indicated by intense fluorescence found at mid-cell (Figure S7b).

Having established that metabolically incorporated and electronically contrasting nitrones can be distinguished from one another, as well as from incorporated azide tag, we next wanted to highlight applications for this rapid dual bioorthogonal labeling method. One key application is the dynamic study of peptidoglycan synthesis through sequential labeling with two UAA probes. To demonstrate this temporal labeling, *L. lactis* or *L. innocua* were incubated in media containing **2**, washed, and then incubated with **1**. After dual SPANC labeling with BCN-CF488 and DBCO-TAMRA, older peptidoglycan was revealed in red and newer peptidoglycan was predominantly yellow (Figures 2, S8, and S9). Older peptidoglycan labeling appears to remain visible along the length of the bacterial cell walls for both strains, with some weak labeling visible at cell poles of *L. lactis*. The newer peptidoglycan appears concentrated at septal planes of *L. lactis*, similar to labeling patterns shown previously for this strain.[30] New peptidoglycan labeling of *L. innocua*, in contrast, resulted in both septal labeling as well as labeling of poles, presumably from cells that had just divided (Figure S9). The rapid dual

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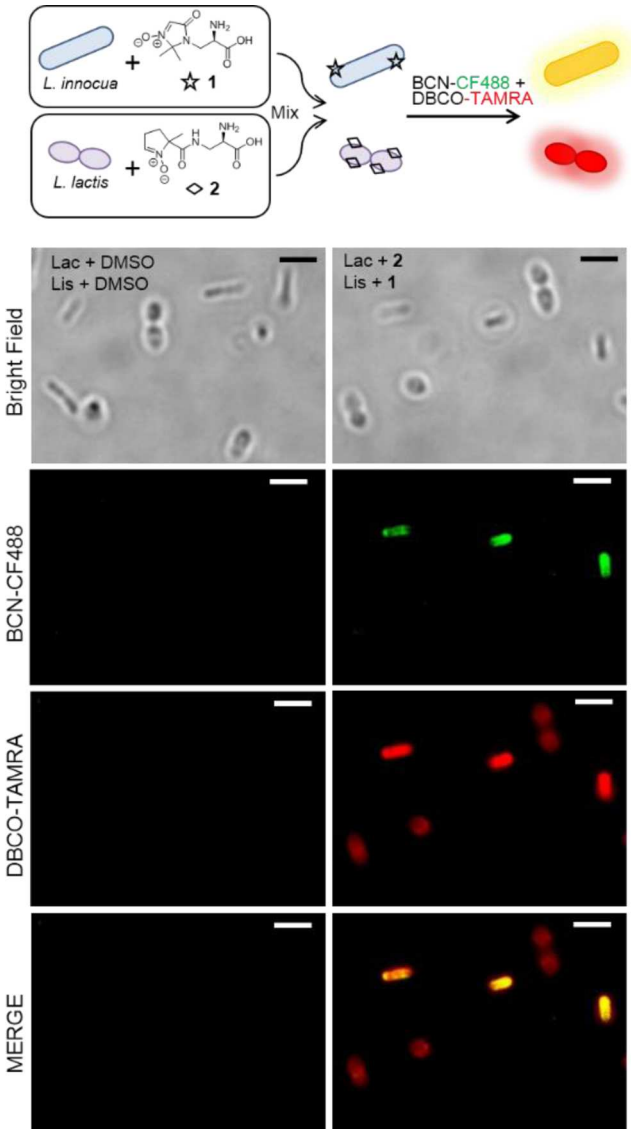

Figure 1. Dual SPANC labeling to distinguish between bacteria incubated with D-alanine functionalized with CMPO or DMImO. *L. lactis* (Lac) were cultured in BHI medium for 1 h at 37 °C with DMSO or 5 mM **2**, whereas *L. innocua* (Lis) were cultured in BHI medium for 1 h at 30 °C with DMSO or 5 mM **1**. Bacteria cultures were then mixed, washed in PBS, and then reacted for 10 min at 37 °C with 25 μM BCN-CF488 and 25 μM DBCO-TAMRA simultaneously. Bacteria were washed again in PBS prior to imaging. Individual cell treatments with either strained alkyne can be found in Figure S5. A wider field of view can be found in Figure S6. Scale bar = 2.5 μm.

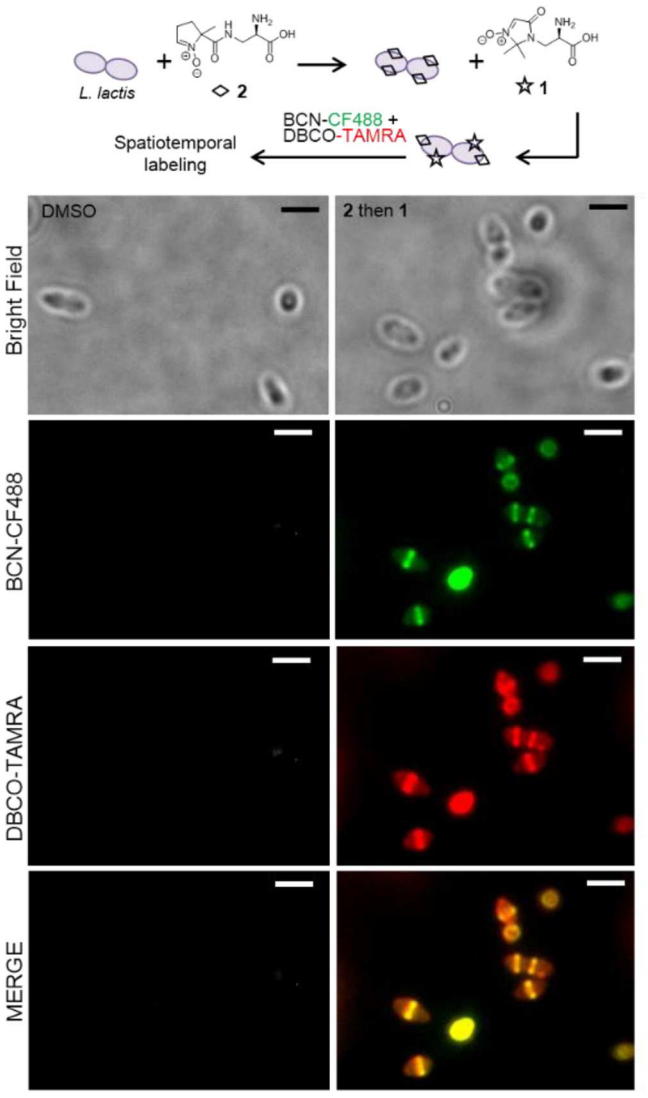

Figure 2. Spatiotemporal labeling of *L. lactis* detected via dual SPANC. *L. lactis* were cultured at 37 °C in BHI medium containing **2** for 10 min, washed in PBS, and then cultured in BHI medium containing **1** for an additional 10 min. Cultures were washed again in PBS and then reacted for 10 min at 37 °C with 25 μM BCN-CF488 and 25 μM DBCO-TAMRA simultaneously. Bacteria were washed again in PBS prior to imaging. Control results with individual labels and reactions with either strained alkyne can be found in Figure S8. Scale bar = 2.5 μm.

SPANC reaction greatly reduces labeling time from alternative sequential-labeling methods and should aid in answering numerous biological questions through further development of nitrone-tagged metabolic precursors and chemical probes.

The ability to detect more than one reactive tag in a single, short bioorthogonal reaction not only is valuable for studying the same biological system but has potential for identifying differences in mixed populations of microorganisms as well. Our initial screening of UAA incorporation and detection via reaction with a fluorescent strained alkyne revealed that *E. coli* were labeled most effectively with **3**, with minimal labeling by the nitrone-tagged UAAs (Figure S3). Given the excellent labeling observed for *L. innocua* with **1** and the preference of *E. coli* to incorporate **3** over the other UAAs, we sought to determine if dual SPAAC/SPANC labeling could discriminate between the two strains grown in a coculture. Both strains of bacteria were cultured together with **1** and **3**, washed, and then reacted with BCN-CF488 and DBCO-TAMRA simultaneously. As shown in Figures 3 and S10, incorporated **1** of *L. innocua* was labeled by BCN-CF488, while *E. coli* with incorporated **3** was revealed by DBCO-TAMRA, resulting in two strains that were easily distinguished from each other through preferential label incorporation and dual labeling detection. It was noted, however, that incorporation of **1** by *L. innocua* was reduced when **3** was present in the coculture (Figure S10a), an effect likely due to competition for similar peptidoglycan biosynthetic pathways. Further development of novel nitrone-tagged probes that target different metabolic pathways, or are selectively incorporated by specific bacterial strains, could lead to powerful

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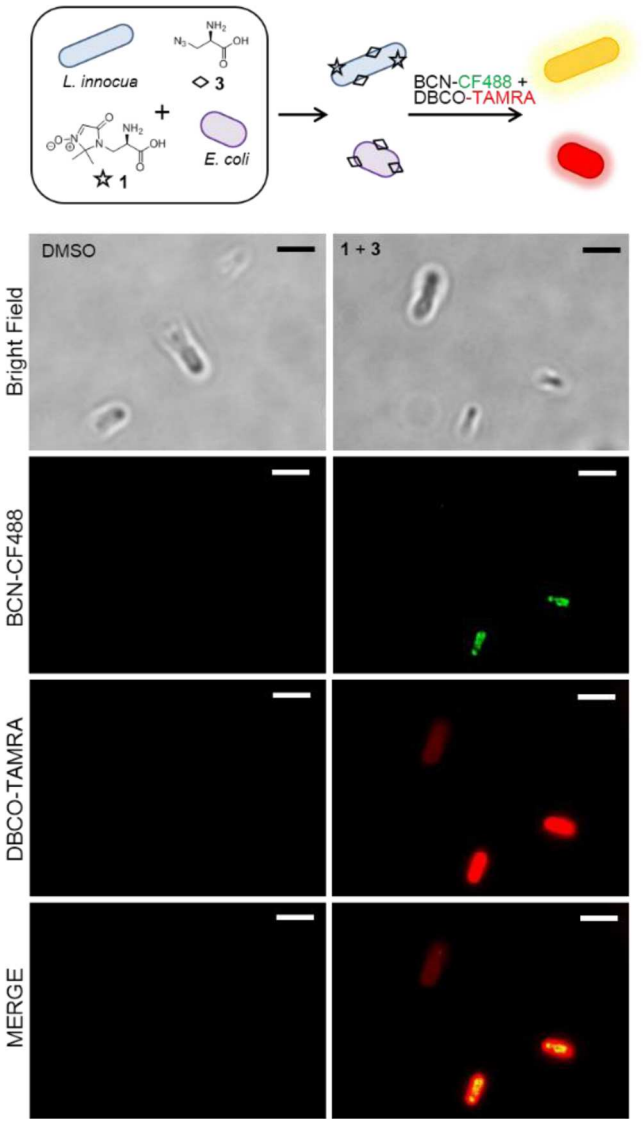

Figure 3. Discrimination of bacterial strains in coculture through dual SPANC/SPAAC. *E. coli* and *L. innocua* were cultured together in the presence of DMSO or both **1** and **3**, washed in PBS, and then reacted for 10 min at 37 °C with 25 μM BCN-CF488 and 25 μM DBCO-TAMRA simultaneously. Control results with individual labels and reactions with either strained alkyne can be found in Figure S10. Scale bar = 2.5 μm.

methods of bacterial identification when combined with dual labeling detection.

Dual SPANC/SPAAC labeling with the reactive pairs described herein has great potential for multiple forms of analysis (e.g., microscopy, in-gel fluorescence, flow cytometry, etc.). However, alternative strained alkyne–nitrone pairs may be necessary for analytical methods such as affinity purification, due to cross reactivity of DBCO with both nitrone tags. Orthogonal reactions with minimal cross reactivity, such as those involving detection of tetrazines or cyclopropenes paired with azides or alkynes,[18−21,23] would also be suitable for such experiments. Furthermore, it is important to acknowledge the potential for side reactions of strained alkynes with thiols,[31] which is problematic for in vitro labeling of cell lysates via strain-promoted reactions. Since the work presented herein focused on labeling viable bacterial cell surfaces, nonspecific strain-promoted labeling was minimal and found to be comparable to cells treated using copper-catalyzed conditions (Figure S11).

CONCLUSION

By careful selection of strained alkyne–nitrone pairs, simultaneous detection of multiple targets is possible through dual SPANC. Orthogonality with azide functionalized probes further expands the potential for dual SPANC/SPAAC reactions to include biological targets that only accept minimalist tags. We have demonstrated multiple applications for this novel dual labeling method, from species discrimination to spatiotemporal labeling of peptidoglycan, all through metabolic incorporation of nitrone and azide-tagged UAAs. The nitrone group has been incorporated into bacterial lipopolysaccharides[32] and has so far proven useful for tagging proteins,[33] antibodies,[11] affinity tags,[32] antibiotics,[27] and fluorophores.[32] Combined with potential for use in applications such as activity-based profiling, for example, which commonly involve bioorthogonal chemistry for detection, the number of possible biological targets for dual labeling is only expanding. Design and synthesis of additional stereoelectronically contrasting nitrone/strained alkyne pairs should also lead to more mutually exclusive reactions for bioorthogonal labeling of living systems such as pathogenic and nonpathogenic prokaryotes.

METHODS

Materials and experimental methods are described in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.6b00063.

Experimental procedures, tables, and supporting figures (PDF)

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Notes

The authors declare no competing financial interest.

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