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Development of whole-cell and cell-free biosensors for the detection and differentiation of organic and inorganic forms of copper†

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The modern world has seen exposure of bacterial communities to toxic metals at selective levels. This manifests itself both intentionally, through medicines and un-intentionally through waste streams. There is growing concern that selective exposure to metals may be linked to microbial resistance to antibiotics. For a microbe to become resistant to a specific metal it must first come in contact with it. The transition metal copper has the ability to enter bacterial cells without need for a copper specific uptake mechanism. Copper is commonly used as an antimicrobial in the healthcare industry, consumer products and as a growth promoter of livestock in the agricultural sector. Here we report a study into the uptake of different organic and inorganic sources of copper. A whole-cell bacterial biosensor was developed to quantify the specific uptake of copper from various sources. Furthermore, a cell-free sensor was utilized to investigate the response to copper sources when uptake is eliminated as a factor. The data within suggest inorganic copper to have greatly reduced uptake compared to organic sources and that there is significant difference between copper oxides, Cu_2O and CuO.

Significance to metallomics

A recent appreciation for the role metals play in bacterial resistance to antibiotics sparked our investigation. We believe that Metallomics is perfect journal to report our findings of metal form contributing to uptake and activity as demonstrated by the whole cell and cell free biosensors developed for this study.

Introduction

The use of antibiotics and metals in healthcare, aquaculture, consumer products and agriculture is common today. With this, bacterial communities are routinely exposed, at selective levels, to these compounds both intentionally, through medicines and unintentionally *via* waste streams.[1] Resistance genes to metals are found in bacteria from diverse environments including those not subject to large-scale human influence. These metal resistance genes sometimes are found together with antibiotic resistance genes on mobile genetic elements.[2] This has led to growing concern that antimicrobial metals could be contributing to antibiotic resistance.[3]

Copper is used in clinical settings, aquaculture, horticulture and in livestock production. Agricultural use of copper includes addition to feed to promote growth by influencing the gut microbiome and to treat/prevent footrot by use in footbaths.[4,5] Report of high copper supplementation in swine feed demonstrating increased levels of copper-resistant isolates suggests that supplementation of copper in animal feed can select for copper resistance.[6] Copper is reportedly able to enter bacterial cells without need for copper specific uptake systems but high concentrations are toxic to the cell.[7] To avoid toxicity due to overexposure to copper, bacteria harbour four systems to maintain copper homeostasis; *cue*, *cus*, *pco* and *cop*. Detoxification and compartmentalization of copper is achieved through charge modification and efflux *via* these systems.[5] Copper as a transition metal has the ability to alternate between oxidation states; cuprous Cu(I) and cupric Cu(II). Bacterial cells can shift the charge of copper from Cu(I) to Cu(II) for toxicity mediated efflux, this led us to the question; does the form in which extracellular copper is present impact its uptake to bacterial communities. To investigate the uptake of copper from diverse sources, a whole-cell biosensor based on the *cue* system was

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developed. This system was adapted to a cell-free biosensor to assess the impact of uptake on sensor readout and provide insights to copper uptake by Gram-negative bacteria.

Experimental section

Plasmid design and construction

Virtual cloning was performed in Benchling [Biology Software] (2019) retrieved from https://benchling.com. pUC19 (Invitrogen) was used as a backbone, the CueR and pCopA sequences were taken from iGEM part (BBa_K198006) and the reporter sfGFP sequence was taken from pJL1-sfGFP (Addgene plasmid # 102634). Insert DNA was ordered as DNA fragments from (GenScript). Plasmid construction was done by Gibson assembly (NEB E5510S) with primers designed using the Benchling Gibson assembly wizard using 20nt overhangs. DNA fragments were PCR amplified using Q5 2X Master Mix (NEB M0492S) using a two-step amplification cycle. PCR products were cleaned using the PureLink Quick Gel Extraction and PCR Purification Kit (Invitrogen K220001) and quantified using a NanoDrop (Invitrogen) prior to use in Gibson Assembly. Sequence verification was done by Sanger sequencing (Eurofins Genomics). The final construct was named pUC19-CueR-pCopAsfGFP-Amp. For the cell-free assay, the constitutive promoter, iGEM part (BBa_J23119), was replaced with a T7 promoter by cutting the construct with NheI and XbaI and sticky end ligation of a phosphorylated oligo duplex, method adapted from ref. 8, to give pUC19-T7CueR-pCopAsfGFP-Amp.

Whole-cell assay

A minimal growth medium comprised of the following was used; 20% 5× M9-Tris salts [10.992 g Tris HCL, 0.5 g NaCl and 1 g NH_3Cl per L], 2% D-glucose [20% w/v solution], casein hydrolysate [20% w/v], 1% thiamine hydrochloride [34 mg L^{-1}], 0.2% $MgSO_4$ [1 M] and $CaCl_2$ [1 M] made up to a final volume with deionized water and supplemented with antibiotic [100 μg mL^{-1} ampicillin sodium salt]. For the cell-based assay (Fig. 1 and 2) a 5 mL overnight culture was inoculated with a single colony of DH5α (Invitrogen 18265017) transformed with pUC19-CueR-pCopAsfGFP-Amp and grown at 37 °C with shaking, 250 rpm. For the assay, 600 μL of overnight culture was used to seed 30 mL cultures and allowed to grow for 2 h at 37 °C with shaking, 250 rpm (outgrowth) prior to addition of copper samples. Prior to use all copper compounds were sterilized by irradiation prior to suspension in assay media. 190 μL of outgrowth culture was added to 10 μL of dilute copper sample (to give final concentrations of; 700, 600, 500, 400, 300, 200, 100, 50, 20, 10, 0.1, 0.01, 0.001, 0.0001 μM) in clear round bottom 96-well plates and grown at 37 °C with shaking, 250 rpm overnight. Following overnight culture, 100 μL of each sample was replace to black-walled, clear bottom 96-well plates for reading. For all samples, optical density at 600 nm (OD_{600}) was read as well as fluorescence with excitation (ex) 485 nm and emission (em) 528 nm to find relative fluorescence units (RFU) using a plate reader (Biotek). All RFU measurements were normalized for cell growth by dividing (RFU/OD_{600}). Limits of 100 (highest reading) and 0 (lowest reading) were used to further normalize results and calculate each growth normalized value on a scale of 0–100. {where RFU/OD_{600} is A; value = $((A - A_{min})/A_{max}) \times 100$}.

Cell-free assay

The Expressway Cell-free *E. coli* Expression kit (Invitrogen K9901-00) was adapted for the purposes of copper detection

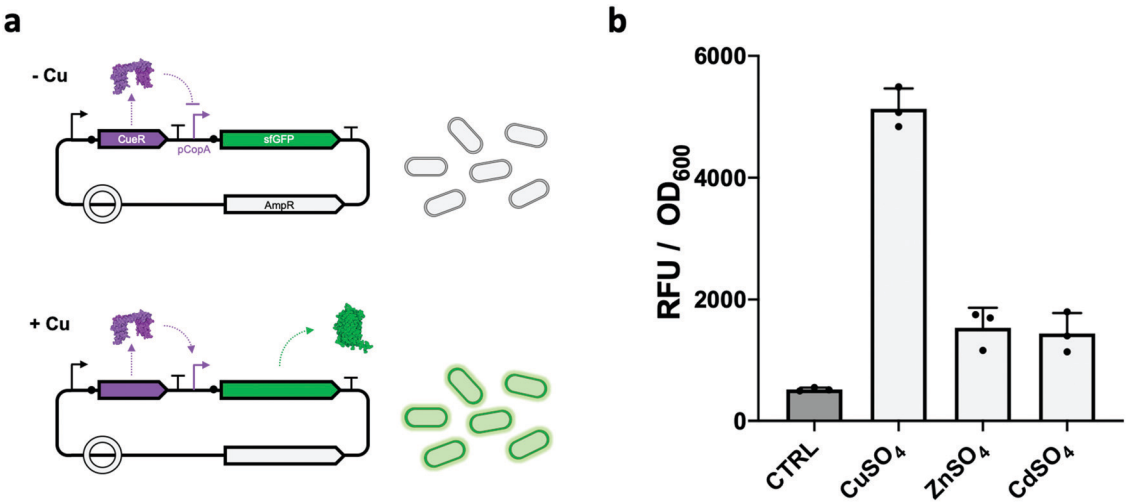

Fig. 1 A whole-cell biosensor for detecting copper uptake. A cell-based biosensor was created for the detection of copper uptake in bacteria. (a) The copper activated transcription factor CueR was driven by a constitutive promoter. In the absence of free Cu(I), CueR acts as a repressor of the pCopA promoter yielding no transcription of the reporter gene sfGFP. The presence of Cu(I) within the cell changes the interaction of CueR and pCopA to promote transcriptional activation of the reporter gene. (b) Transformed *E. coli* DH5α were grown in M9-Tris media spiked with 100 μM of $CuSO_4$, $ZnSO_4$ or $CdSO_4$ and the reporter output monitored with respect to a no spike control (CTRL). Statistical analysis was done by one-way ANOVA with Dunnett's multiple comparisons test, stars indicate statistical difference with respect to the no copper (CTRL), ($p \leq 0.05$*, 0.01**, 0.001***, 0.0001****). Cartoons of 3-D protein structures were generated using Illustrate.[20]

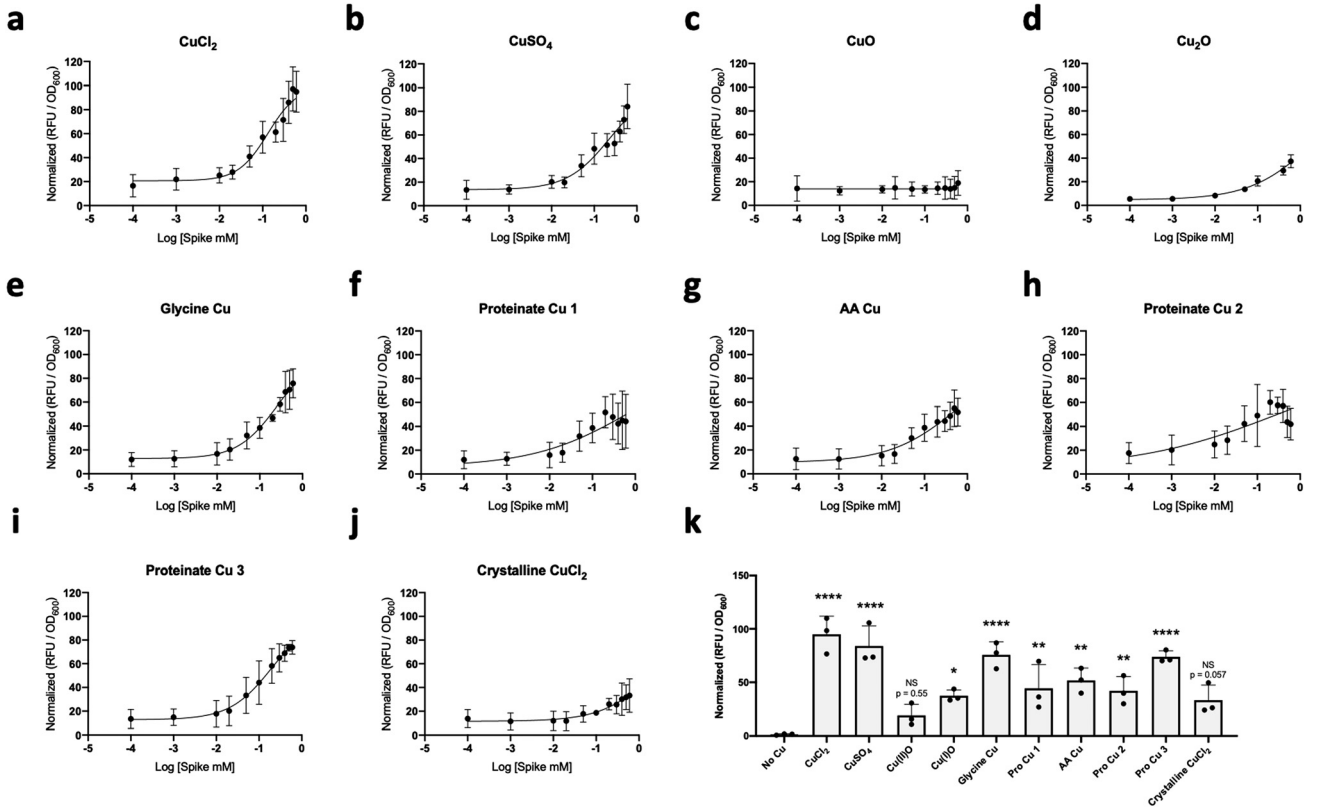

Fig. 2 Whole-cell detection of copper uptake from diverse sources. Cells transformed with the copper sensor construct were grown in the presence of various dietary copper supplements; (a) $CuCl_2$, (b) $CuSO_4$, (c) CuO, (d) Cu_2O, (e) Gu-Glycine, (f) Cu-Proteinate, (g) Cu-amino acid hydrolysate, (h) Cu-Proteinate, (i) Cu-Proteinate, (j) crystalline $CuCl_2$. Relative fluorescence units (RFU) were recorded 16 h post spike with copper samples, [1–0.0001 mM] and normalized to culture growth, optical density at 600 nm (OD_{600}). These values were set to a 100-scale with respect to the highest value recorded. Error is shown as the standard deviation of three replicate cultures run on different days. (k) The response to each copper source at a spike of 600 μM plotted as a histogram. Data is represented as the mean of three replicate experiments performed on different days. Error bars represent the standard deviation of replicates. Statistical analysis was done by one-way ANOVA with Dunnett's multiple comparisons test, stars indicate statistical difference with respect to the no copper (No Cu) control, ($p \leq 0.05$*, 0.01**, 0.001***, 0.0001****).

using the construct pUC19-T7CueR-pCopAsfGFP-Amp. An initial master mix with; 4 μL *E. coli* slyD – Extract, 4 μL 2.5× IVPS reaction buffer, 0.25 μL 50 mM amino acids (–Met), 0.2 μL 75 mM methionine, 0.2 μL T7 RNAP, 0.2 μg DNA template made up to 10 μL final volume per reaction. The initial reaction was incubated at 37 °C with shaking, 300 rpm for 30 min. Following this, 10 μL of the master mix was added to wells of a 384-well black-walled clear bottom plate. Each reaction was supplemented with an equal volume of the feed mix from the Expressway cell-free kit; per reaction; 5 μL 2× IVPS Feed Buffer, 0.25 μL 50 mM amino acids (–Met), 0.2 μL 75 mM methionine, 20 μM copper sample made up to 10 μL final volume per reaction. The 20 μL reactions were incubated at 30 °C for 4 h with shaking, 300 rpm. RFU, ex 485 nm and em 528 nm was measured on a plate reader (Biotek).

Statistical analysis

Statistical analysis was carried out using Prism version 8.3.0 (GraphPad) and tests used for specific experiments are indicated in the accompanying figure legend.

Data availability

All data generated for this study is present in the manuscript and raw data files will be made available upon reasonable request.

Results

Development of whole-cell biosensor for copper uptake detection

The *cue* system is a primary response to elevated environmental copper levels in *E. coli*.[9] The *cue* system sees the expression of the copper efflux pump CopA which removes Cu(I) from the cytosol to the periplasm[7,10] and an oxidase, CueO, which catalyses the aerobic oxidation of Cu(I) to Cu(II) in the periplasm.[11,12] The expression of CopA and CueO is controlled by the MerR-like metalloregulatory transcription factor CueR.[10,13–15] CueR binds directly the *copA* promoter pCopA.[9] Knowing this, a copper sensing expression construct was designed to elucidate the uptake of copper by *E. coli in vivo* (Fig. 1a). CueR is driven by a strong constitutive promoter (pJ23119, iGEM part BBa_J23119) with a reporter gene, sfGFP,

under the transcriptional control of the CueR activated promoter pCopA. CueR has been reported to have sensitivity to copper, silver and gold but is not responsive to zinc.[13–16] *E. coli* DH5α transformed with pUC19-CueR-pCopAsfGFP-Amp were grown under normal minimal media conditions (CTRL), see methods, along with exposure to a source of copper ($CuSO_4$), zinc ($ZnSO_4$) and cadmium ($CdSO_4$) (Fig. 1b). Overnight exposure to 100 μM of metal showed specificity of the sensor to copper over zinc and cadmium, as seen by strong induction of the sfGFP reporter (Fig. 1b).

Evaluating uptake of copper from diverse sources

Knowing that it was possible to specifically detect the uptake of copper using *E. coli* transformed with the CueR – pCopA-reporter, the sensor was used to determine the uptake capacity of cells when exposed to diverse sources of copper potentially found in the environment resulting from agricultural waste. Copper-sensor transformed DH5α were exposed to diverse copper sources at defined concentrations in minimal media overnight. Four controls were used; $CuCl_2$, $CuSO_4$, CuO and Cu_2O (Fig. 2a–d) along with a number of chelated copper sources commonly used as nutritional supplements in the animal-feed industry; Cu-Glycine (Fig. 2e), Cu-Amino acid hydrolysate (Fig. 2g), Cu-Proteinates (Fig. 2f, h, and i) and crystalline $CuCl_2$ (Fig. 2j). A comparison of reporter output at 600 μM exposure shows a significant reduction in the uptake of CuO (p = 0.0244) and Cu_2O (p = 0.0272) with respect to $CuCl_2$ (Fig. 2k). Noteworthy was the difference in response between the two copper oxides with CuO exposed cells showing no response (Fig. 2c) yet Cu_2O inducing the reporter with exposure above 200 μM (Fig. 2d). Affirmation of the copper sample specific uptake was observed by analysis of the culture OD_{600} with respect to copper spike (Fig. S1, ESI†). Samples failing to elicit a strong induction of the sensor; Cu_2O, CuO and crystalline Cu had little to no drop in culture OD_{600} with respect to spiked sample concentration. Failure of the sample to be uptaken by the cell in these cases did not result in a drop in culture OD_{600} due to a combination of copper toxicity or translational burden of copper induced reporter.

Cell-free activity of copper sources

Having observed differences in response to copper source from a whole-cell biosensor, we investigated if this difference in response could be attributed to cellular uptake. To achieve this, uptake was eliminated as a factor by implementation of a cell-free detection system. For this a near identical genetic sensor was used, with just a single change. The promoter driving the expression of CueR was substituted for a T7 promoter to give the construct pUC19-CueR-T7pCopAsfGFP-Amp. This was used with the Expressway *E. coli* cell-free expression kit to develop a cell-free copper sensing assay (Fig. 3a). Cell-free transcription–translation reactions were performed as per manufacture's protocol with one addition. Post 30 minute pre-incubation, the feed solutions were spiked with 10 μM of copper samples and incubated at 30 °C for 4 h (Fig. 3b). A significant reduction in reporter output with exposure to CuO was found when compared with both $CuCl_2$ (p = 0.005) and $CuSO_4$ (p = 0.0231).

Discussion

Elevated levels of environmental copper has been linked with generation of copper-resistant strains.[6] With growing concern that bacterial metal resistance can co-select for antibiotic resistance[3] we set out to investigate if the form in which a metal is present in a bacterial population's local environment could impact its uptake and subsequent ability to stimulate expression of heavy metal resistance elements. The whole-cell

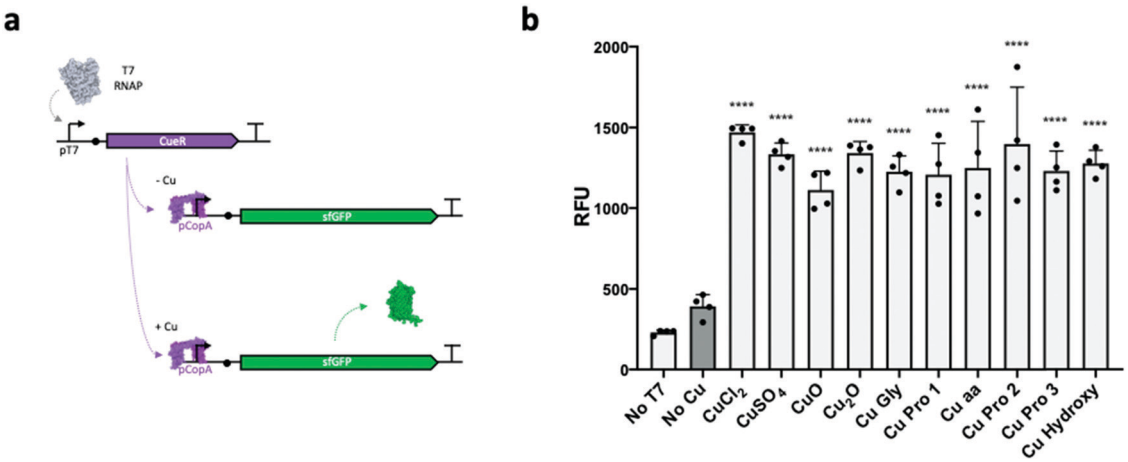

Fig. 3 Cell-free evaluation of copper source. A cell-free expression system was used to evaluate the impact of copper samples on gene activation by CueR on pCopA when cellular uptake is eliminated as a factor. (a) CueR expression was driven by a T7 promoter with user defined addition of T7 RNA polymerase. (b) Cell-free reactions were spiked with copper samples at a final concentration of 10 μM and incubated overnight (12–16 h) at 37 °C. Data is represented as the mean of four replicate experiments performed on different days. Error bars represent the standard deviation of replicates. Statistical analysis was done by one-way ANOVA with Dunnett's multiple comparisons test, stars indicate statistical difference with respect to the no copper (No Cu) control, ($p \leq 0.0001$****).

biosensor developed for this investigation effectively induced reporter production in the presence of copper over the other metals tested, zinc and cadmium (Fig. 1b). Stimulation of the sensor by zinc and cadmium was surprising given *in vitro* reports suggesting no cross-reactivity with zinc.[13–16] We suggest this observed activity could be a background cellular response to increased zinc and cadmium as zinc is known to play an integral role in ribosome function[17] and cadmium toxicity can lead to stress induced translational up-regulation.[18] Elevated levels of zinc have also been suggested to cross-talk with copper homeostatic pathways.[19] Future experiments could be done to address this surprising observation such as evaluation of reporter production from a constitutive promoter in the presence or absence of metal or incorporation of a constitutive reporter into the metal reporter construct.

Evaluation of copper sources in the whole-cell biosensor revealed disparity in induction of the copper responsive reporter between organic and inorganic forms of the element. Both controls; $CuCl_2$ and $CuSO_4$ showed the highest dynamic range. The copper containing animal feed supplements displayed a similar mean in sensor output at 600 μM (Fig. 2k). Inorganic copper samples; CuO, Cu_2O and crystalline Cu showed poor induction of whole-cell sensor reporter with respect to organic sources, with CuO failing to elicit a response. Cu_2O did however elicit a statistically significant, (p = 0.0252), upregulation in reporter production. Taken together these data suggest that there is not only a difference in the uptake of organic and inorganic metal containing solutes but that the uptake of metal within these designations is highly variable. To confirm that these observations where indeed due to uptake, sample uptake was eliminated as a factor. The genetic construct designed for the whole cell biosensor was adapted for use with a commercial bacterial cell-free transcription–translation kit. In using such a system it was possible to assess the response of the copper sensor to all organic and inorganic samples. In this case all samples tested elicited a significant signal in the cell-free system (Fig. 3b). This further supports the sample dependent uptake finding of this study. It may be interesting to further validate the findings of biosensor stimulation through direct measurement of copper in the lysates of cells grown in the presence of these copper sources tested. This could provide even greater insight to the mechanisms of uptake of these copper sources.

In summation we have developed a bacterial whole-cell biosensor for the detection of copper from various organic and inorganic forms. Our results suggest that there is significant disparity in the uptake between organic and inorganic forms of delivery. Furthermore, the copper oxidation states, cuprous (Cu_2O) and cupric (CuO), can alter the ability of the metal to be up-taken. As bacterial cells can shift the charge of copper from Cu(I) to Cu(II) for toxicity mediated efflux, it makes sense that CuO supplemented media did not stimulate the whole-cell biosensor. It will be interesting to investigate if such a result is the case across a panel of microbial hosts and if oxides of other metals have similarly low up-take.

Author contributions

Experimental design and research was done by AC, MC and RM. Design, construction and testing of the genetic constructs was done by AC. Sourcing of copper sources was done by JP and RM. Data analysis and manuscript preparation was a collaborative effort between AC, MC and RM.

Conflicts of interest

Two authors, JP and RM are employed by Alltech, a manufacturer of organic copper proteinates.

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