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Comparative Mechanisms of Protein Transduction Mediated by Cell-Penetrating Peptides in Prokaryotes

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Abstract Bacterial and archaeal cell envelopes are complex multilayered barriers that serve to protect these microorganisms from their extremely harsh and often hostile environments. Import of exogenous proteins and nanoparticles into cells is important for biotechnological applications in prokaryotes. In this report, we demonstrate that cell-penetrating peptides (CPPs), both bacteria-expressed nona-arginine peptide (R9) and synthetic R9 (SR9), are able to deliver noncovalently associated proteins or quantum dots into four representative species of prokaryotes: cyanobacteria (*Synechocystis* sp. PCC 6803), bacteria (*Escherichia coli* DH5α and *Arthrobacter ilicis* D-50), and archaea (*Thermus aquaticus*). Although energy-dependent endocytosis is generally accepted as a hallmark that distinguishes eukaryotes from prokaryotes, cellular uptake of uncomplexed green fluorescent protein (GFP) by cyanobacteria was mediated by classical endocytosis. Mechanistic studies revealed that macropinocytosis plays a critical and major role in CPP-mediated protein transduction in all four prokaryotes. Membrane damage was not observed when cyanobacterial cells were treated with R9/GFP complexes, nor was cytotoxicity detected when bacteria or archaea were treated with SR9/QD complexes in the presence of macropinocytic inhibitors. These results indicate that the uptake of protein is not due to a compromise of membrane integrity in cyanobacteria, and that CPP can be an effective and safe carrier for membrane trafficking in prokaryotic cells. Our investigation provides important new insights into the transport of exogenous proteins and nanoparticles across the complex membrane systems of prokaryotes.

Keywords Archaea · Bacteria · Cell-penetrating peptides (CPPs) · Cyanobacteria · Endocytosis · Macropinocytosis

Abbreviations

BFP	Blue fluorescent protein
CPP	Cell-penetrating peptide
CytD	Cytochalasin D
EIPA	5-(*N*-ethyl-*N*-isopropyl)-amiloride
GFP	Green fluorescent protein
MTT	1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan
NEM	*N*-ethylmaleimide
OPH	Organophosphorus hydrolase
QD	Quantum dot
R9	Bacteria-expressed nona-arginine
RFP	Red fluorescent protein
SR9	Synthetic nona-arginine
Wort	Wortmannin

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Introduction

The bacterial cell envelope is a complex multilayered barrier that serves to protect microorganisms from their unpredictable and often hostile environment (Silhavy et al. 2010). Gram-positive bacteria are delimited by a single membrane (monoderm), and are surrounded by a thick

peptidoglycan cell wall outside the membrane. Gram-negative bacteria are double membrane-enveloped (diderm) microorganisms that contain three principal layers in the envelope: an outer membrane, a thin peptidoglycan cell wall, and an inner (or cytoplasmic) membrane. Gram-negative cyanobacteria are photo-autotrophic prokaryotes (Ruffing 2011) that possess a special envelope structure: cell wall (including surface layer, outer membrane bilayer, and peptidoglycan layer) and cytoplasmic membrane (Peschek 1984). The outer membrane comprises molecules with high content of negatively charged groups that confers a negative surface charge and is devoid of electron transport. In some species, this outer membrane may be completely covered by a surface layer that contains proteins or glycoproteins. The unusually thick peptidoglycan wall, ranging from 10 to 200 nm, confers rigidity to the cell morphology. The cytoplasmic membrane of cyanobacteria is the site of solute transport, as in all living cells, and forms an osmotic barrier between the cell interior and the surrounding medium. In addition to the envelope structure, cyanobacteria possess layers of highly specialized thylakoid membranes, also called intracytoplasmic membranes, located in the cytosol (Pfeil et al. 2014). Cyanobacterial thylakoid membranes have attached phycobilisomes (light-harvesting complexes) and make contact with the plasma membrane. Thylakoid membranes are the major sites of photosynthetic light reactions and respiratory electron transport (Mullineaux 2014). In green algae and land plants, however, thylakoid membranes are organized in grana stacks and are located inside the chloroplasts (Pfeil et al. 2014).

The division of life forms into three major domains of cellular life, Archaea, Bacteria, and Eukarya, is now widely accepted (Woese and Fox 1977). It is generally accepted that Archaea and Eukarya are sister groups (Caetano-Anolles et al. 2014). A characteristic feature that really separates archaea apart from the other two domains of life is the chemical composition of the cytoplasmic membrane (Bolhuis 2004). Bacterial and eukaryal membranes contain mostly phospholipids that are derived from fatty acids linked to glycerol by an ester bond. In contrast, archaeal lipids are composed of saturated phytanyl chains that are linked to glycerol by an ether bond (Bolhuis 2004). Archaeal membranes consist of phospho-, glyco-, and phospho-glyco-lipids (Oger and Cario 2013). Some archaea, in particular thermophiles and acidophiles, have tetraether lipids that span the entire membrane and form a monolayer, instead of a bilayer (Bolhuis 2004). Archaeal lipids are very stable, which probably enables archaea to tolerate extremely harsh and hostile environments (Bolhuis 2004; Oger and Cario 2013).

The import of exogenous proteins into bacteria was rarely studied (Chungjatupornchai and Fa-aroonsawat 2009). Nevertheless, protein import across bacterial envelope structure becomes quite important as its understanding would accelerate advancement of synthetic biology, as well as biotechnological applications, in bacteria.

Cell-penetrating peptides (CPPs), also known as protein transduction domains (PTDs) or arginine-rich intracellular delivery (AID) peptides, are short peptides with usually less than 30 amino acids that possess the ability to penetrate plasma membranes (Fonseca et al. 2009; van den Berg and Dowdy 2011; Futaki et al. 2013; Chang et al. 2014). The first CPP was discovered in a transcriptional transactivator, transactivator of transcription (Tat), of the immunodeficiency virus type 1 (HIV-1) (Frankel and Pabo 1988; Green and Loewenstein 1988). Subsequently, many natural or synthetic CPPs were discovered or chemically synthesized with common compositions of cationic, amphipathic, or hydrophobic amino acids (Fonseca et al. 2009; van den Berg and Dowdy 2011). Not only can CPPs enter cells by themselves, but they also can facilitate delivery of various cargoes into cells. This phenomenon is sometimes called protein transduction (van den Berg and Dowdy 2011; Chang et al. 2014). Versatile CPPs can deliver a wide range of cargoes, including nucleic acids, proteins, liposomes, nanomaterials, and pharmaceuticals. CPPs, bacteria-expressed nona-arginine (R9) or synthetic R9 (SR9), can efficiently deliver noncovalently complexed macromolecules into animal and plant cells in fully active forms (Chang et al. 2014). The advantages of noncovalent attachment in CPP-mediated transduction include ease of use, ease of production, and versatility with respect to both cargo composition and functional delivery.

Although the precise mechanisms underlying CPP-mediated cellular internalization remain controversial, two major routes of CPP transduction have recently been identified: direct membrane translocation and endocytosis-mediated entry (Futaki et al. 2013; Chang et al. 2014). Factors that influence the entry routes of CPPs into cells include amino-acid compositions of the CPPs, CPP concentrations, cell type, zeta-potential of the CPP-cargo complexes, and particle size (van den Berg and Dowdy 2011; Chang et al. 2014). Endocytic pathways can be further categorized into classical endocytosis, clathrin- and caveolin-dependent endocytosis, and macropinocytosis (Conner and Schmid 2003; Dump and Dowdy 2007). We previously demonstrated that macropinocytosis is the major route for cellular internalization of noncovalently associated R9/cargo complexes (Chang et al. 2007; Hou et al. 2007; Liu et al. 2008, 2010). Recently, we demonstrated that cyanobacteria use classical endocytosis and macropinocytosis to internalize exogenous green fluorescent protein (GFP) and R9/GFP complexes, respectively

(Liu et al. 2008, 2013a). Accordingly, CPPs which utilize various internalization pathways are valid tools for studies of cellular uptake of materials which affect diverse cellular processes. In this report, we compare the cellular uptake mechanisms for CPP-mediated protein transduction in four representative prokaryotes, namely Gram-negative *Synechocystis* sp. PCC 6803 cyanobacteria and *E. coli* DH5α, Gram-positive *Arthrobacter ilicis* D-50 bacteria, and *Thermus aquaticus* archaea.

Advances in the field of nanotechnology have led to remarkable breakthroughs in the area of microbial diagnosis (Syed 2014). Quantum dots (QDs) are semiconducting nanocrystals that exhibit a size-dependent tunable photoluminescence (Brucher et al. 1998; Chan and Nie 1998). QDs possess a number of advantages over traditional dyes, including a high quantum yield, a narrow range of emission wavelength, long photostability, and high extinction coefficient (Michalet et al. 2005). Consequently, QDs have been used successfully for the detection of *Cryptosporidium parvum* (a protozoan), Gram-negative *Escherichia coli* O157, *Salmonella typhimurium*, and Gram-positive *Mycobacterium* spp. (Huang et al. 2014). In the present study, both fluorescent proteins and QDs were used to monitor CPP-mediated cellular internalization in prokaryotes.

Materials and Methods

Cell Culture

For bacterial culture, *Synechocystis* sp. PCC 6803 (American Type Culture Collection, Manassas, VA, USA; 27184) was grown in BG-11 medium with mild shaking at 50 rpm and regular illumination at 28 °C (Liu et al. 2013a). Both *E. coli* DH5α (Invitrogen, Carlsbad, CA, USA) and *A. ilicis* D-50 strains were grown in 3 ml of Luria–Bertani (LB) broth at 37 °C without any antibiotics, until the bacterial suspension reached an optical density at 600 nm (OD_{600}) of 0.5, as previously described (Liu et al. 2008).

For archaeal culture, *T. aquaticus* (ATCC, 25105) was pre-cultured with #461 broth (ATCC) in tubes at 70 °C. The culture tubes were incubated within a closed jar with a moistened paper towel to maintain humidity. Once a high density of bacteria was reached, 200 µl of cell suspension was transferred into a new flask with fresh #461 broth, and the culture continued (Liu et al. 2008).

Plasmid, Protein, Peptide, and QD Preparation

The pR9 and pRFP plasmids contain coding sequences of R9 and red fluorescent protein (RFP) under the control of the T7 promoter, respectively (Chang et al. 2007). The pQE8-GFP plasmid consists of a coding sequence for GFP under the control of the T5 promoter (Chang et al. 2005). Plasmid DNA was prepared and purified using the Nucleobond AX100 Kit (Machery-Nagel, Duren, Germany).

For protein expression, plasmids were individually transformed into *E. coli*, as previously described (Chang et al. 2005, 2007). Expressed R9 fusion proteins were then purified using metal-chelate affinity chromatography, concentrated using Amicon Centriprep YM-30 (Millipore, Billerica, MA, USA), and quantified using the Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

SR9 containing nine-arginine residues was chemically synthesized to a purity of 94 % using high-performance liquid chromatography (HPLC) and confirmed by mass spectrometry (Genomics, Taipei, Taiwan) (Liu et al. 2010).

Carboxyl-functionalized CdSe/ZnS QDs (eFluor 525NC) with a maximal emission peak wavelength at 525 nm were purchased from eBioscience (San Diego, CA, USA) (Liu et al. 2013b).

Noncovalent Protein Transduction

To prepare the complexes, 2.4 µM of R9 peptide was incubated with 800 nM of either GFP or RFP at a molecular ratio of 3:1 at room temperature for 10 min (Liu et al. 2013a). To investigate the internalization of exogenous proteins, various types of cells, including cyanobacteria, DH5α and D-50 bacteria, and archaea were washed with double-deionized water and treated with their specific media as the negative controls. Cyanobacteria, DH5α, and D-50 were exposed to either uncomplexed GFP at a final concentration of 800 nM or noncovalent R9/GFP complexes prepared at a molecular ratio of 3:1 for 1 h. For archaea, either uncomplexed RFP at a final concentration of 800 nM or R9/RFP complexes prepared at a molecular ratio of 3:1 were added to cells for 20 min (Liu et al. 2008).

To prepare the other complexes, 48 µM of SR9 peptide was incubated with 800 nM of QD at a molecular ratio of 60:1 at room temperature for 1 h (Liu et al. 2010). To investigate the internalization of exogenous QDs, SR9/QD complexes prepared at a molecular ratio of 60:1 were incubated with DH5α, D-50 bacteria, or archaea for 1 h.

To identify the mechanisms of exogenous protein internalization in cyanobacteria, we used physical and pharmacological inhibitors that perturb membrane transport processes. They were 2 mM *N*-ethylmaleimide (NEM) (Sigma-Aldrich, St. Louis, MO, USA), 10 µM fusicoccin (Sigma-Aldrich), 2 µM nigericin (Fluka Chemie, Seelze, Germany), 2 µM valinomycin (Sigma-Aldrich), and incubation at a low temperature (4 °C) (Liu et al. 2008, 2013a). To study macropinocytosis, cyanobacteria were treated with 100 µM 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) (Sigma-Aldrich), 10 µM cytochalasin D (CytD) (Sigma-Aldrich), or 100 nM wortmannin (Wort) (Sigma-Aldrich),

followed by the exposure to R9/GFP complexes (Liu et al. 2008, 2013a). CytD stops the F-actin rearrangement and disrupts all forms of endocytosis, including clathrin-, caveolae-dependent endocytosis, and macropinocytosis (Liu et al. 2010, 2011). EIPA is a specific macropinocytic inhibitor that inhibits the Na^+/H^+ exchange (Liu et al. 2010). Wort inhibits macropinocytosis by interrupting the action of phosphoinositide 3-kinase (Hansen et al. 1995).

Fluorescent and Confocal Microscopy

For fluorescent and bright-field images, cells were recorded using an Eclipse E600 fluorescent microscope (Nikon, Melville, NY, USA) with a Penguin 150CL cooled CCD camera (Pixera, Los Gatos, CA, USA), an AE31 fluorescent microscope (Motic, Causeway Bay, Hong Kong) with an IS1000 eyepiece (Tucsen, Fujian, China), or an Olympus BX51 fluorescent microscope with a DP50 cooled CCD camera (Olympus, Center Valley, PA, USA), as previously described (Liu et al. 2013a, c).

For confocal images, cells were recorded with both TCS SL and SP5 II confocal microscope systems (Leica, Wetzlar, Germany). The parameters of a TCS SL confocal microscopy were set as follows: excitation at 488 nm and emission at 500–530 nm for the detection of GFP, and excitation at 543 nm and emission at 580–650 nm for the detection of RFP. The parameters of a TCS SP5 II confocal microscopy were set as follows: excitation at 405 nm and emission at 436–480 nm for the detection of blue fluorescent protein (BFP), excitation at 488 nm and emission at 498–523 nm for the detection of GFP, and excitation at 594 nm and emission at 630–680 nm for the detection of autofluorescence in red. Intensities of fluorescent images were quantified using the UN-SCAN-IT software (Silk Scientific, Orem, UT, USA) (Liu et al. 2008, 2013a).

Membrane Leakage Analysis

To analyze the cytotoxicity of R9/GFP complexes and endocytic inhibitors in cyanobacteria, membrane leakage was analyzed using a dual fluorescence reporter assay (Liu et al. 2013a). Cyanobacteria were treated with BG-11 medium as a negative control, treated with 100 % methanol as a positive control, or treated with R9/GFP complexes in the presence of endocytic modulators for 24 h. After washing with double-deionized water, cells were stained with 5 μM of either SYTO 9 (LIVE/DEAD BacLight Bacterial Viability Kit, Molecular Probes, Eugene, OR, USA) or SYTOX blue (Invitrogen) for 30 min at room temperature. SYTO 9 stains nucleic acids of both live and dead prokaryotes in green fluorescence. SYTOX blue only stains the nucleic acids of membrane-damaged cells in bright blue fluorescence. After washing with double-deionized water, cyanobacteria were observed using an Olympus BX51 fluorescent microscope.

1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) Assay

To assess the cytotoxicity of SR9/QD complexes, DH5α, D-50 bacteria, and archaea were treated with their media as negative controls, while prokaryotes were treated with 75 % alcohol (EtOH) as positive controls. DH5α, D-50 bacteria, and archaea were treated with SR9/QD complexes prepared at a molecular ratio of 60:1 in the absence or presence of macropinocytic inhibitors, including CytD, EIPA, and Wort for 1 h. The MTT assay was performed as previously described (Liu et al. 2013d).

Statistical Analysis

Results are expressed as mean ± standard deviation (SD). Mean values and SDs were calculated from at least three independent experiments carried out in triplicates for each treatment group. Statistical comparisons were performed by ANOVA or the Student's *t* test, using levels of statistical significance of $P < 0.05$ (*, α) and $P < 0.01$ (**, αα), as indicated.

Results

Protein Transduction in Prokaryotes

Protein Transduction in Gram-Negative Bacteria

To assess protein transduction in Gram-negative bacteria, both *Synechocystis* sp. PCC 6803 cyanobacteria and *E. coli* DH5α bacteria were treated with either uncomplexed fluorescent protein or R9/fluorescent protein complexes. Uncomplexed GFP could be spontaneously delivered into cyanobacteria (Fig. 1a), as were R9/GFP complexes. In bacteria, there was no autofluorescence observed in Gram-negative and Gram-positive bacteria (data not shown), while red autofluorescence was detected in cyanobacteria (Fig. 1a, the bottom row). These results are consistent with our previous finding (Liu et al. 2008, 2013a) that both uncomplexed GFP and R9/GFP complexes can be taken up by cyanobacteria.

Remarkably, DH5α treated with R9/GFP complexes displayed green fluorescence, but only slight green fluorescence when treated with uncomplexed GFP (Fig. 1b). This indicates that uncomplexed GFP cannot spontaneously enter DH5α, but the protein complexed with R9 can be transported into DH5α. When DH5α bacteria were treated with either uncomplexed QD or SR9/QD

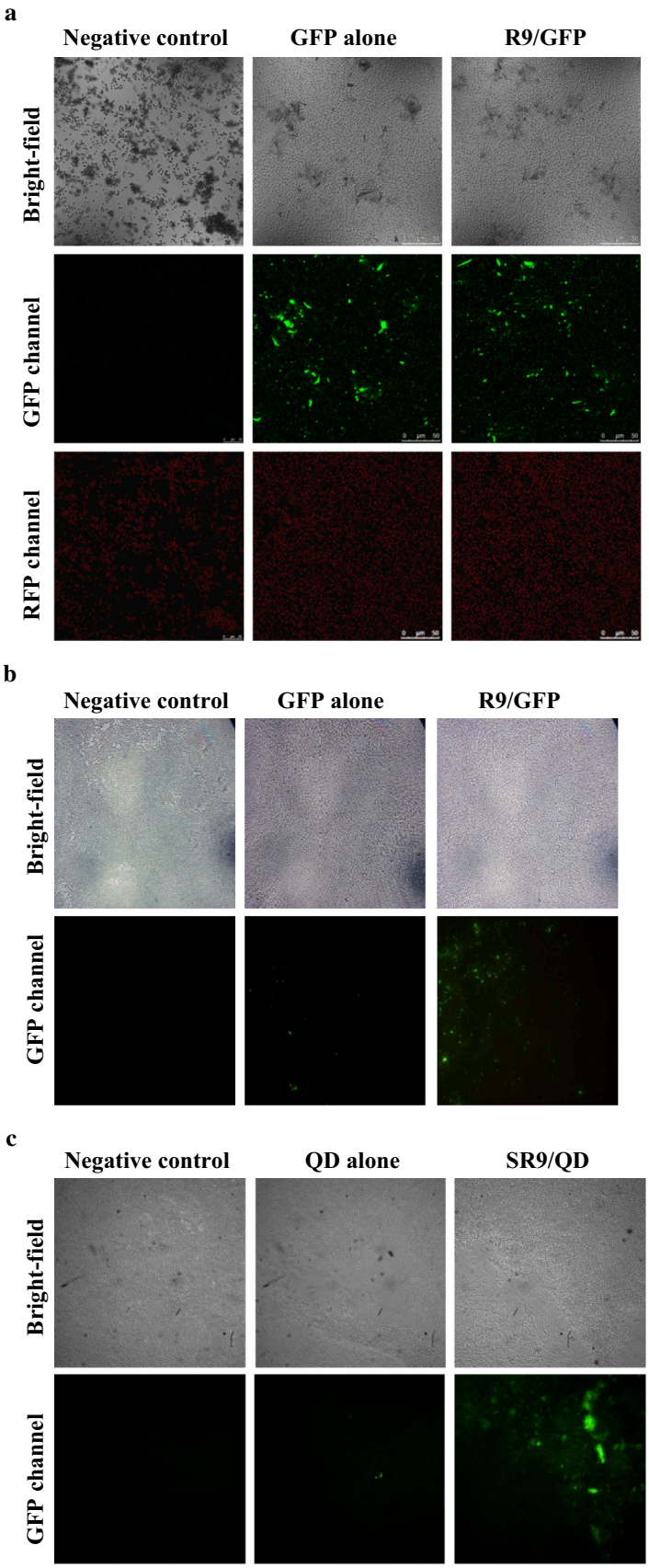


Fig. 1 Protein transduction in Gram-negative bacteria. **a** Protein transduction and autofluorescence in *Synechocystis* sp. PCC 6803 cyanobacteria. Cells were treated with medium (negative control), uncomplexed GFP, or R9/GFP complexes. Protein transduction was recorded with both bright-field and GFP channel, while cell autofluorescence was examined with RFP channel, using a confocal microscope (TCS SP5 II, Leica). *Scale bar* is 50 μm. **b** Protein transduction in *E. coli* DH5α bacteria. Cells were treated with medium, uncomplexed GFP, or R9/GFP complexes, and observed with both bright-field and GFP channel using a fluorescent microscope (Nikon). **c** Protein transduction in *E. coli* DH5α bacteria. Cells were treated with medium, uncomplexed QD, or SR9/QD complexes, and observed with both bright-field and GFP channel using a fluorescent microscope (Motic)

complexes, little fluorescent signal was observed in DH5α treated with uncomplexed QD (Fig. 1c), but strong fluorescent signal was detected in DH5α treated with SR9/QD complexes. These data support the notion that R9 can deliver exogenous nanoparticles into DH5α.

Protein Transduction in Gram-Positive Bacteria

Gram-positive *A. ilicis* D-50 bacteria were treated with uncomplexed GFP, QD, R9/GFP, or SR9/QD complexes. D-50 treated with R9/GFP complexes (Fig. 2a) or SR9/QD complexes (Fig. 2b) displayed green fluorescence, while little green fluorescence was observed in bacteria treated with uncomplexed GFP or QD. These results indicate that R9/GFP and SR9/QD complexes can be delivered into D-50.

Protein Transduction in Archaea

T. aquaticus archaea were treated with uncomplexed RFP, QD, R9/RFP, or SR9/QD complexes. In archaea, no autofluorescence was observed when the cells treated with medium (Fig. 3a). Slight fluorescence was observed in the group treated with uncomplexed RFP (Fig. 3a) or QD (Fig. 3b). In contrast, bright red fluorescence was observed in the cells treated with R9/RFP (Fig. 3a) or SR9/QD (Fig. 3b) complexes, indicating that CPPs can facilitate the delivery of RFP and QD into archaea. Collectively, our results demonstrated that CPP-mediated protein transduction exists in the domains Bacteria and Archaea. Additionally, only cyanobacteria spontaneously take up exogenous proteins, and subsequent experiments were focused on the study of its mechanism.

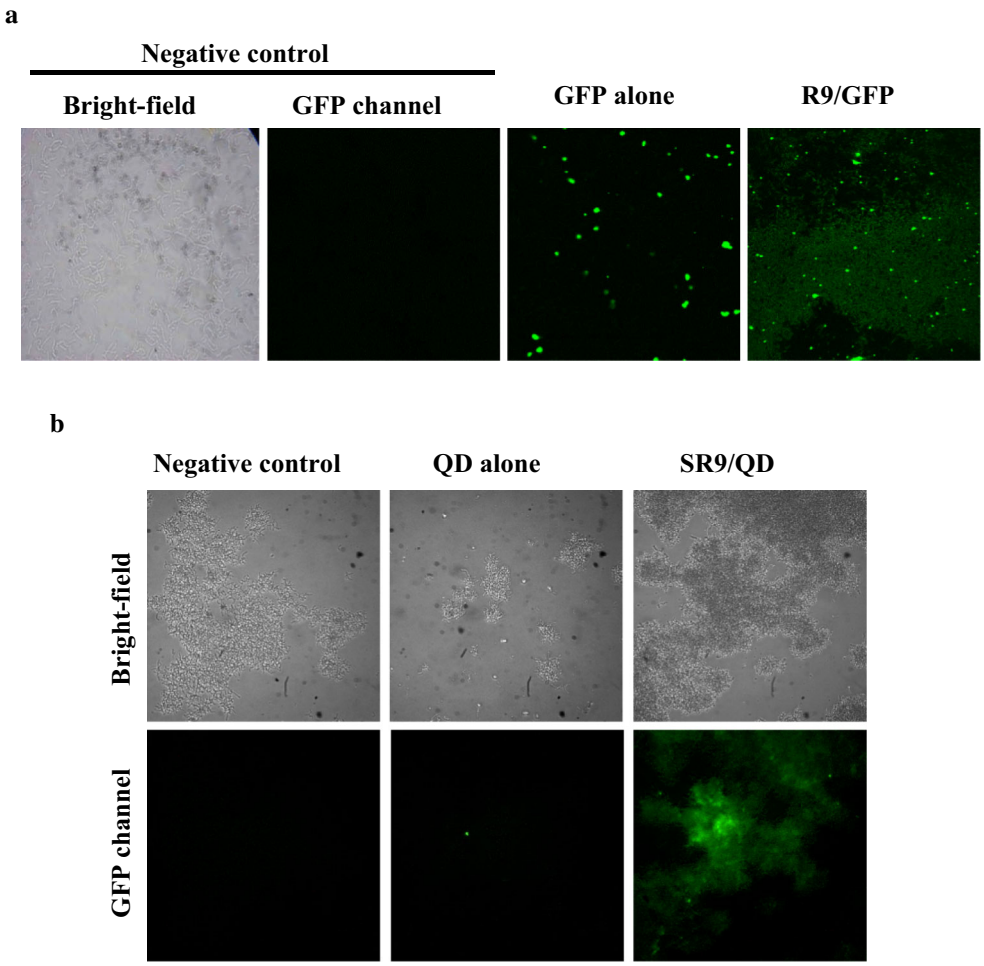


Fig. 2 Protein transduction in Gram-positive bacteria. **a** Protein transduction in *A. ilicis* D-50 bacteria. Cells were treated with medium as a negative control, uncomplexed GFP, or R9/GFP complexes, and observed using fluorescent (negative control, Nikon) and confocal microscopes (Leica). **b** Protein transduction in D-50 bacteria. Cells were treated with medium (negative control), uncomplexed QD, or SR9/QD complexes, and observed with both bright-field and GFP channel using a fluorescent microscope (Motic)

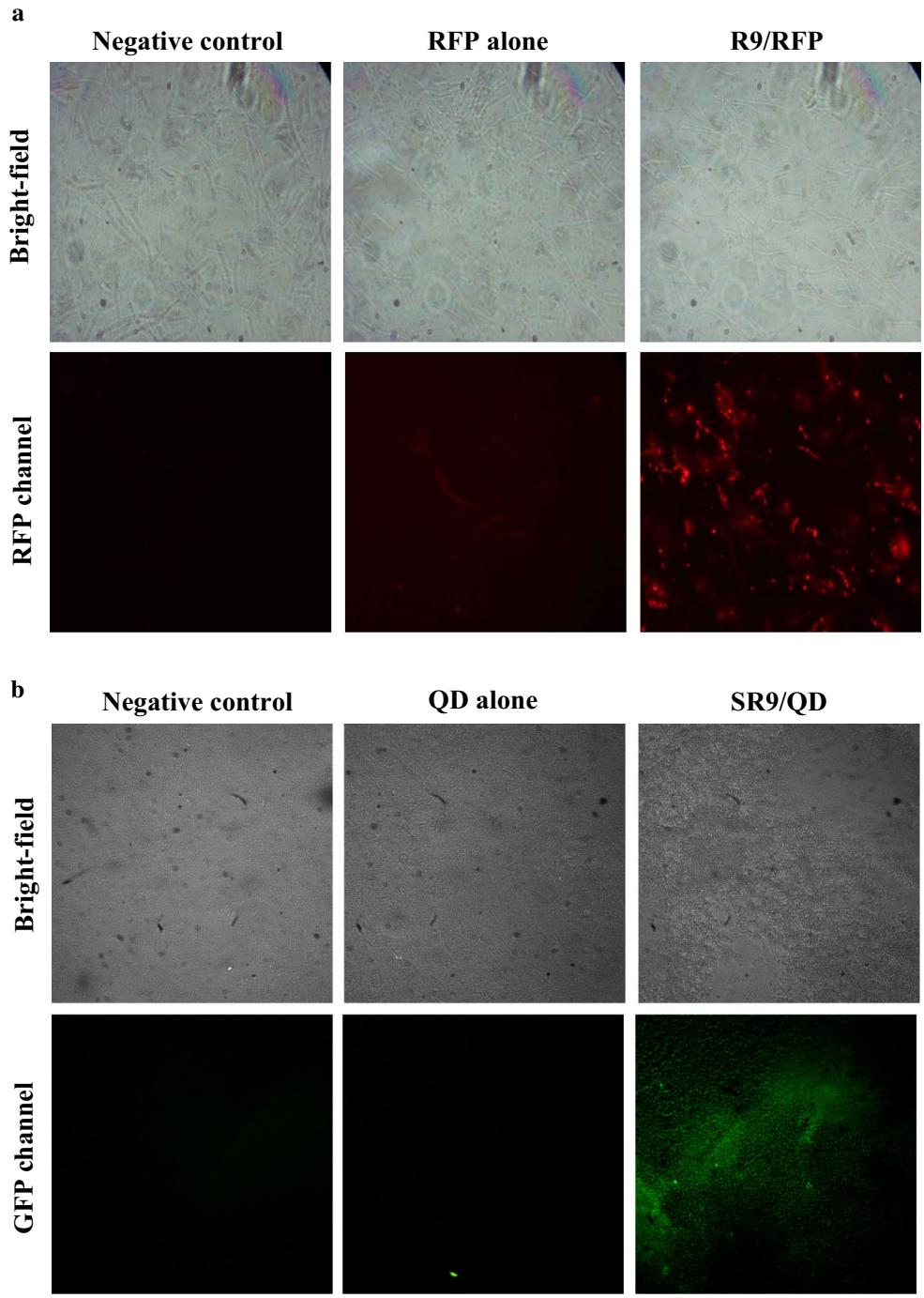


Fig. 3 Protein transduction in archaea. **a** Protein transduction in *T. aquaticus* thermophiles. Archaea were treated with medium as a negative control, uncomplexed RFP, or R9/RFP complexes, and observed with bright-field and RFP channel using a fluorescent microscope (Nikon). **b** Protein transduction in archaea. Cells were treated with medium (negative control), uncomplexed QD, or SR9/QD complexes, and observed with both bright-field and GFP channel using a fluorescent microscope (Motic)

The Mechanism of Spontaneous Protein Internalization in Cyanobacteria

To study the mechanism of spontaneous protein uptake in cyanobacteria, cells were treated with GFP in the presence or absence of physical and pharmacological inhibitors of membrane transport processes, including NEM, fusicoccin, nigericin, valinomycin, low temperature (4 °C) for complete energy depletion, and sodium azide. Entry of GFP into cyanobacteria was reduced by all of endocytic

inhibitors (Fig. 4a). Among these treatments, NEM was the most effective, blocking more than 90 % of GFP internalization (Fig. 4b). These data confirm that classical endocytosis is the major route for spontaneous internalization of uncomplexed GFP in cyanobacteria. Accordingly, NEM was used to block classical endocytosis in subsequent experiments.

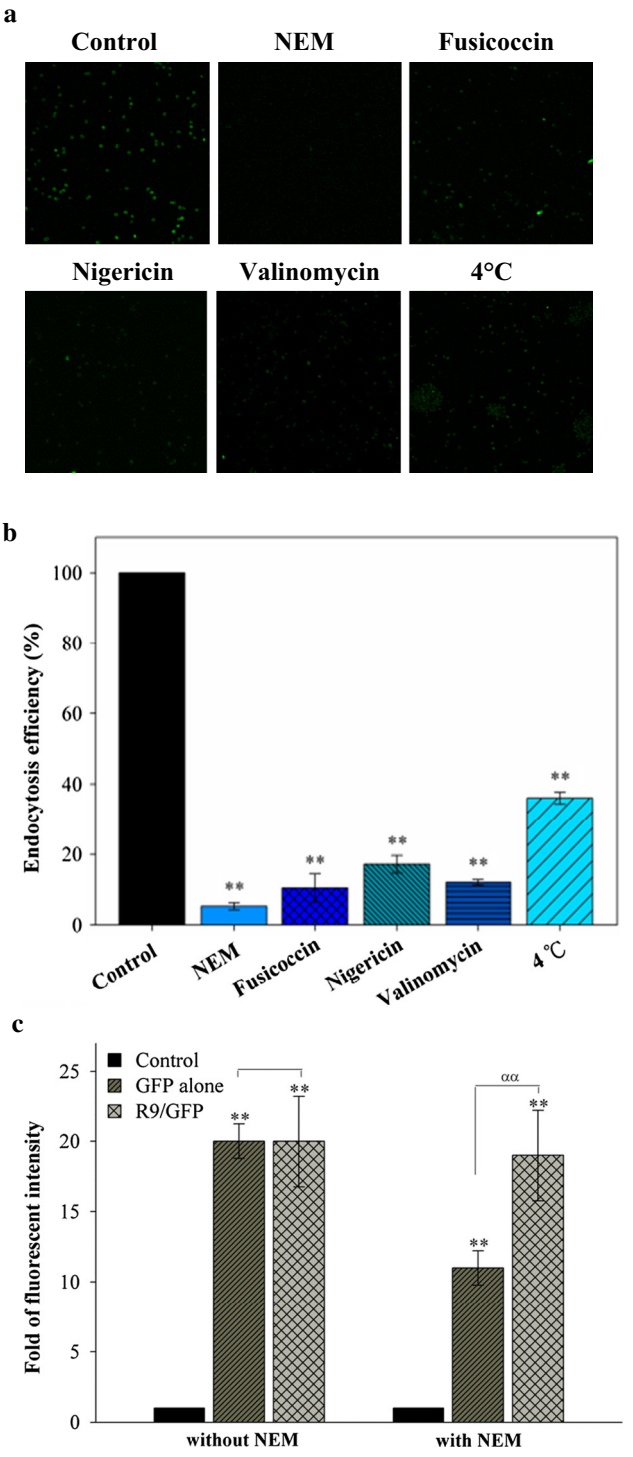
To understand the effect of NEM on CPP-mediated protein penetration, cyanobacteria were treated with medium, uncomplexed GFP, or R9/GFP complexes in the absence or presence of 2 mM NEM. Without NEM, uncomplexed GFP and R9/GFP complexes exhibited similarly high internalization efficiencies (Fig. 4c). In contrast, NEM treatment decreased endocytic efficiency in cyanobacteria treated with uncomplexed GFP but not in cyanobacteria treated with R9/GFP complexes. This indicates that classical endocytosis is a major mechanism for uptake of uncomplexed GFP, but not for CPP-mediated protein transduction. Accordingly, we hypothesize there is another major route for protein transduction in cyanobacteria that does not involve classical endocytosis.

The Mechanism of CPP-Mediated Protein Transduction in Cyanobacteria

To identify the major route for cellular entry of R9/GFP complexes in cyanobacteria, cells were treated with R9/GFP complexes in the presence or absence of NEM and macropinocytic inhibitors, including CytD, EIPA, and Wort, as indicated (Fig. 5a). To analyze the effects of classical endocytic and macropinocytic modulators, fluorescent images were quantified. All of these macropinocytic inhibitors reduced protein transduction in cyanobacteria in both the absence and the presence of NEM (Fig. 5b). Among these treatments of macropinocytic inhibitors, EIPA, a specific macropinocytic inhibitor, was the most efficient inhibitor of protein transduction in the absence of NEM. However, CytD and Wort caused significant inhibition in the presence of NEM. Taken together, these results suggest that both classical endocytosis and macropinocytosis contribute to the entry of R9/GFP complexes. Moreover, lipid raft-dependent micropinocytosis, rather than classical endocytosis, appears to play the major and critical role in the transduction of R9/GFP complexes in cyanobacteria.

Mechanisms of CPP-Mediated Protein Transduction in DH5α, D-50 Bacteria, and Archaea

To study the internalization mechanisms of SR9/QD complexes in prokaryotes, DH5α, D-50, and archaea were treated with SR9/QD complexes in the presence or absence of macropinocytic inhibitors, including CytD, EIPA, and Wort. All of these macropinocytic inhibitors significantly inhibited protein transduction in DH5α (Fig. 6a), D-50 (Fig. 6b), and archaea (Fig. 6c). These results indicate that macropinocytosis also plays a major role in CPP-mediated protein transduction of SR9/QD complexes in these prokaryotes.



◀**Fig. 4** The mechanism of uncomplexed GFP internalization in cyanobacteria. **a** Effects of endocytic inhibitors on GFP uptake by cyanobacteria. Cells were treated with 800 nM of uncomplexed GFP either in the absence of endocytic inhibitors (Control) or in the presence of 2 mM NEM, 10 μM fusicoccin, 2 μM nigericin, 2 μM valinomycin, or at low temperature (4 °C), as indicated. **b** Histogram of the effects of endocytic inhibitors on GFP uptake by cyanobacteria. The results depicted in **a** were quantified using UN-SCAN-IT software. **c** Effects of NEM on protein transduction in cyanobacteria. Cells were treated with medium, uncomplexed GFP, or R9/GFP complexes, as indicated, in the absence or presence of NEM. Significant differences from control at $P < 0.05$ (*, α) and $P < 0.01$ (**, αα) are indicated. Data are presented as mean ± SD from five independent experiments in each treatment group

Membrane Leakage of CPP-Mediated Protein Transduction in Cyanobacteria

To confirm all treatments were not cytotoxic and that protein transduction of R9/GFP complexes did not result from nonspecific membrane damage, membrane leakage was assessed. Cells were treated with BG-11 medium and 100 % methanol as negative and positive controls, respectively (Fig. 7). Cells were treated with R9/GFP complexes in the presence of endocytic inhibitor (NEM) or macropinocytic inhibitors (CytD, EIPA, or Wort), as indicated. Red autofluorescence indicated functional chlorophyll in

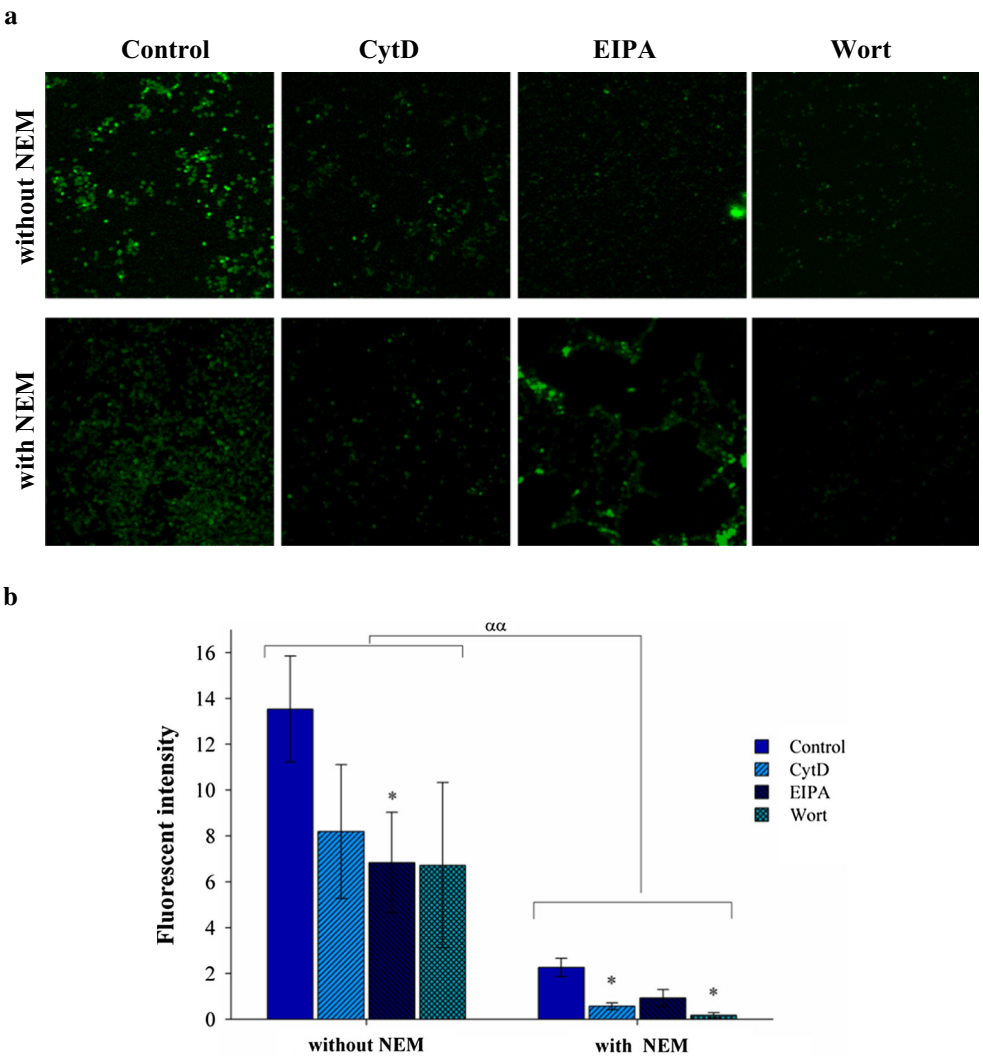
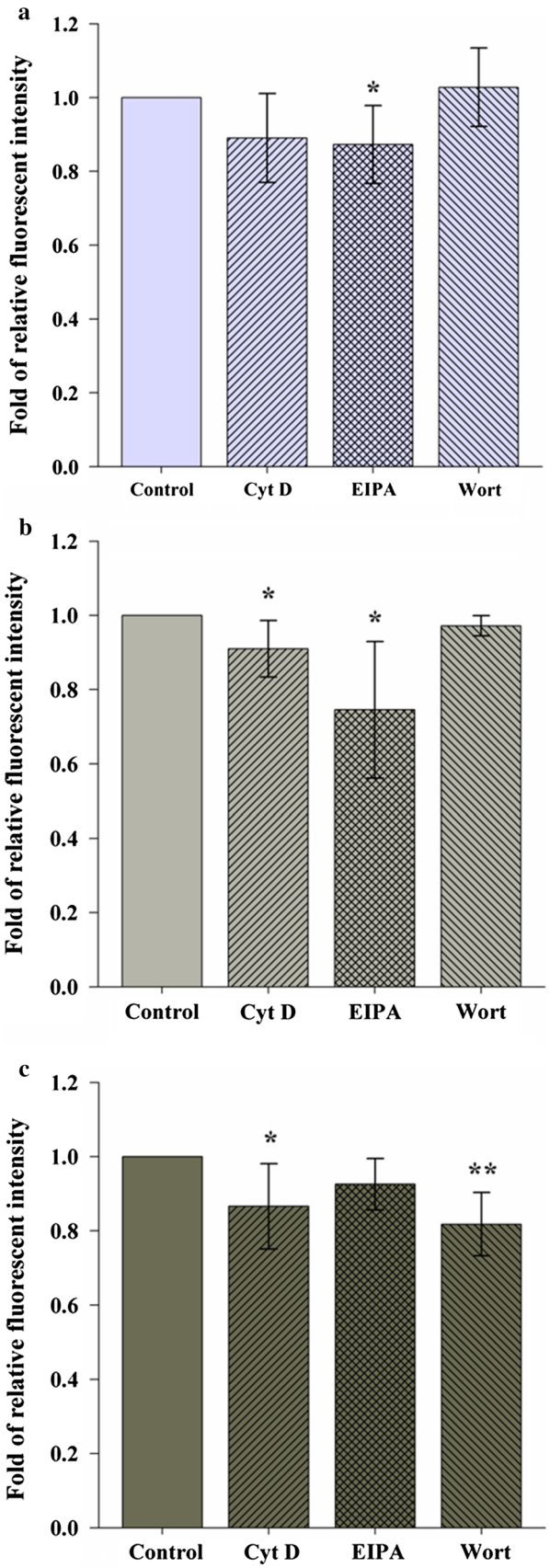


Fig. 5 The mechanism of CPP-mediated protein transduction in cyanobacteria. **a** Effects of macropinocytic inhibitors in cyanobacteria treated with or without NEM. Cells were treated with R9/GFP complexes in the absence or presence of NEM and the macropinocytic inhibitors CytD, EIPA, and Wort, as indicated. Cells treated without any macropinocytic modulators served as the control. Green fluorescence was observed using a confocal microscope (TCS SL, Leica). **b** Histogram of the effects of macropinocytic inhibitors on protein transduction in cyanobacteria treated with or without NEM. Results from (**a**) were quantified using UN-SCAN-IT software. Significant differences from control at $P < 0.05$ (*, α) and $P < 0.01$ (**, αα) are indicated. Data are presented as mean ± SD from five independent experiments in each treatment group



◀**Fig. 6** Cellular internalization mechanisms of CPP-mediated protein transduction in prokaryotes. DH5α (**a**), D-50 (**b**), and archaea (**c**) were treated with SR9/QD complexes in the absence (controls) or presence of various macropinocytic inhibitors (CytD, EIPA, or Wort, as indicated). Cells were observed with GFP channel using a fluorescent microscope (Motic). Green fluorescence intensity were quantified using UN-SCAN-IT software. Significant differences from control at $P < 0.05$ (*) and $P < 0.01$ (**) are indicated. Data are presented as mean ± SD from five independent experiments in each treatment group

live cyanobacteria, while blue fluorescence and lack of red autofluorescence indicated damaged/dead cells. There was no evidence of membrane damage associated with CPP-mediated protein transduction in cyanobacteria.

Cytotoxicity of CPP-Mediated Protein Transduction in DH5α, D-50 Bacteria, and Archaea

To determine the effect of the transduction of CPP/QD complexes in the presence of macropinocytic inhibitors on cell viability, the MTT assay was performed. Transduction of SR9/QD complexes did not reduce cellular growth caused in either the absence or presence of macropinocytic inhibitors in DH5α (Fig. 8a), D-50 (Fig. 8b) bacteria, or archaea (Fig. 8c). These data indicate that CPP should be a safe vehicle for carry cargo into prokaryotes.

Discussion

In this report, we demonstrate that nontoxic CPP-mediated protein transduction is possible in four representative species/strains of prokaryotes: cyanobacteria (*Synechocystis* sp. PCC 6803), bacteria (*E. coli* DH5α and *A. ilicis* D-50), and archaea (*T. aquaticus*). Lipid raft-dependent macropinocytosis plays the major role in CPP-mediated protein transduction of SR9/QD complexes in DH5α, D-50 bacteria, and archaea. In contrast, both classical endocytosis and macropinocytosis contribute to exogenous protein import into *Synechocystis* sp. PCC 6803 cyanobacteria: uptake of uncomplexed GFP by cyanobacteria is mediated by classical endocytosis, while internalization of CPP-mediated protein transduction involves macropinocytosis. Our data are consistent with other studies of bioactive macromolecule delivery in prokaryotes (Table 1) (Liu et al. 2008, 2013a) and eukaryotes (Chang et al. 2007, 2014; Hou et al. 2007; Huang et al. 2015). Accordingly, CPPs represent a potentially useful, nonclassical endocytosis-mediated, trafficking system across membranes in cyanobacteria.

The presence of a highly differentiated membrane system, comprising an outer membrane, peptidoglycan

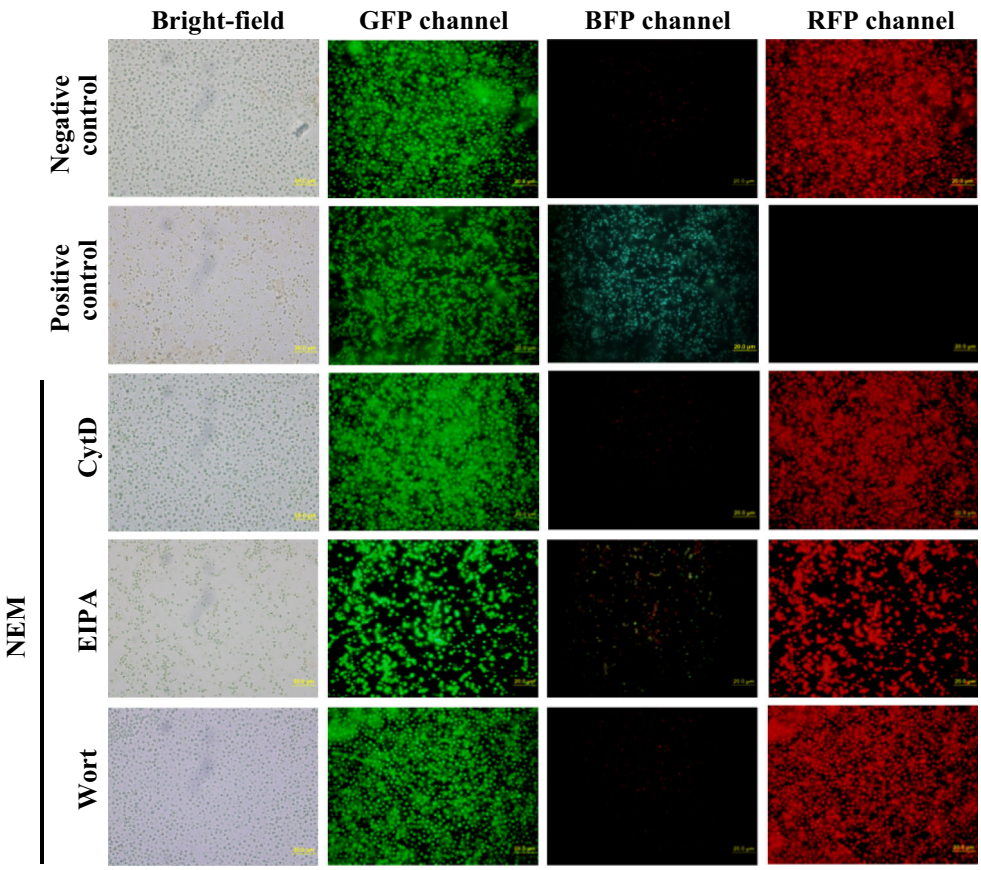
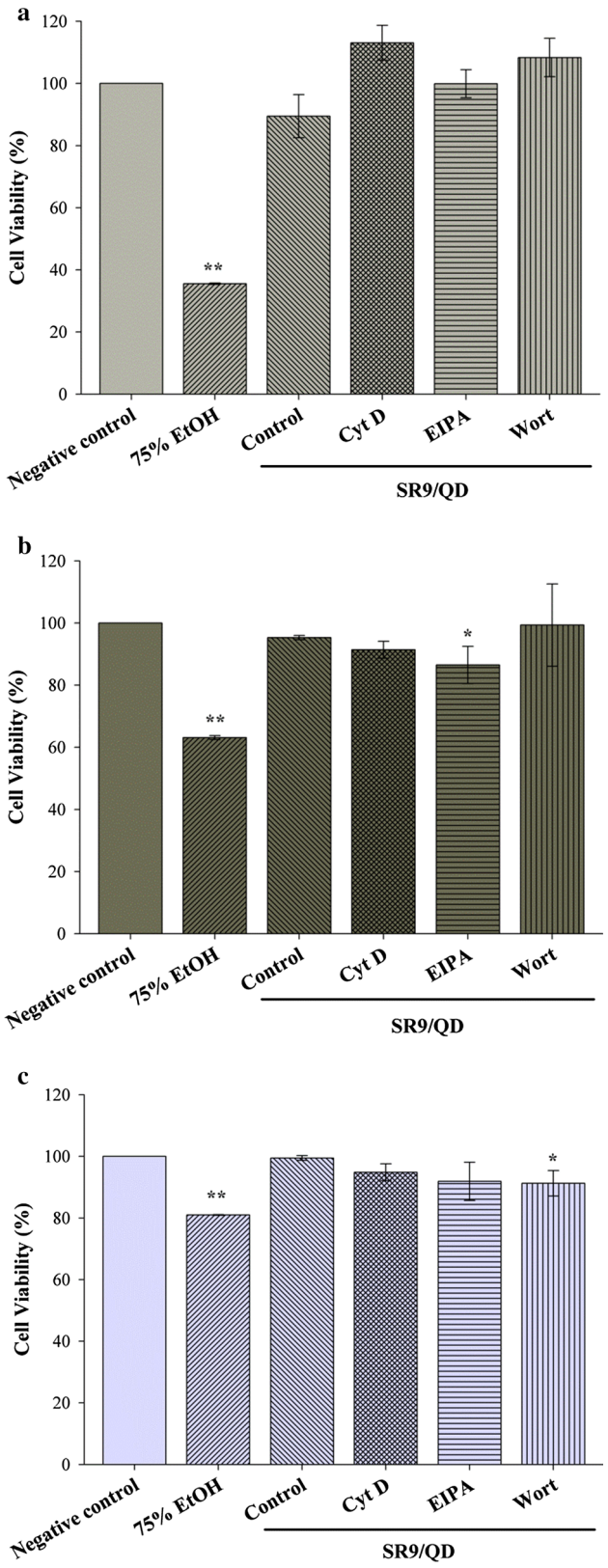


Fig. 7 Membrane leakage of cyanobacteria treated with R9/GFP complexes in the presence of endocytic and macropinocytic inhibitors. Cyanobacteria were either treated with BG-11 medium as a negative control, or 100 % methanol as a positive control. In the presence of the endocytic inhibitor NEM, cells were treated with R9/GFP complexes in the presence of various macropinocytic inhibitors (CytD, EIPA, or Wort, as indicated). Membrane leakage analysis was conducted in GFP and BFP channels using a dual-color fluorescent measurement: SYTO 9 stains nucleic acids of both live and dead prokaryotes in green fluorescence, while SYTOX blue only stains the nucleic acids of membrane-damaged cells in bright blue fluorescence. Cyanobacterial autofluorescence was observed in the RFP channel. Images were recorded using a fluorescent microscope (Olympus). *Scale bar* is 20 μm

layer, and cytoplasmic membrane, renders cyanobacteria a unique among bacteria (Chungjatupornchai and Fa-a-roonsawat 2009). The targeting of proteins across cyanobacterial membrane systems presents a major challenge. Exogenous proteins are targeted into or across a single plasma membrane in most bacteria (Spence et al. 2003). However, cyanobacteria face the problem of targeting proteins through two entirely different membranes (outer membrane and cytoplasmic membrane) separated by a thick peptidoglycan layer. Our understanding of exogenous protein transport into cyanobacteria is limited because (1) no in vitro assay is available, and (2) no protein translocation mutants have been isolated (Chungjatupornchai and Fa-aroonsawat 2009). Mackle and Zilinskas attempted to target the chloramphenical acetyltransferase (CAT) to the periplasmic space and thylakoid lumen of *Synechococcus elongatus* PCC 7942 cyanobacteria using various signal peptides (Mackle and Zilinskas 1994), as listed in Table 1. Spence et al. then reported a direct translocation of GFP to the periplasmic space of *Synechocystis* sp. PCC 6803 cyanobacteria using a bacterial twin arginine translocation pathway (Spence et al. 2003). Recently, Chungjatupornchai and Fa-aroonsawat targeted organophosphorus hydrolase (OPH) to the outermost cell surface and cell wall (Chungjatupornchai and Fa-aroonsawat 2008), and GFP to the periplasm (Chungjatupornchai and Fa-aroonsawat 2009) of *S. elongatus* PCC 7942 cyanobacteria using an anchoring motif of a bacterial ice nucleation protein (Inp). Chungjatupornchai et al. also demonstrated a translocation of OPH to the outermost cell surface of *S. elongatus* PCC 7942 cyanobacteria using an anchoring motif of *Synechococcus* outer membrane protein A (SomA) (Chungjatupornchai et al. 2011).

Energy-dependent endocytosis has long been a hallmark to distinguish eukaryotes from prokaryotes (Lonhienne et al. 2010). This energy-dependent process is an eukaryote-specific membrane-trafficking system (de Duve 2007; Doherty and McMahon 2009). Protein transport across cytoplasmic membranes in bacteria has long been thought to involve only export of proteins to the exterior of the cell (Fuerst and Sagulenko 2014). Endocytosis, while universal among eukaryotes, had not been identified in Bacterial or Archaeal domain (Lonhienne et al. 2010). Lonhienne et al. initially discovered protein uptake in bacteria of the *planctomycetes Gemmata obscuriglobus* by an energy-dependent endocytosis-like process (Lonhienne et al. 2010). The internalized proteins were



◀**Fig. 8** Cytotoxicity of CPP-mediated protein transduction in prokaryotes. Cells were treated with their specific media and 75 % alcohol (EtOH) as negative and positive controls, respectively. DH5α (**a**), D-50 (**b**) bacteria, and archaea (**c**) were treated with SR9/QD complexes in the absence (controls) or presence of the macropinocytic inhibitors CytD, EIPA, or Wort, as indicated. Cell viability was analyzed using the MTT assay. Significant differences from control at $P < 0.05$ (*) and $P < 0.01$ (**) are indicated. Data are presented as mean ± SD from five independent experiments in each treatment group

located in endomembrane-like compartments and degraded in the paryphoplasm. A previous report of thylakoid membrane in cyanobacteria obliquely confirmed that endomembrane system and membrane trafficking might be possible (Liberton et al. 2011). Recently, our data showed that cyanobacteria use classical endocytosis and macropinocytosis to internalize uncomplexed GFP and R9/GFP complexes, respectively (Liu et al. 2013a). Notably, our present results demonstrate that CPP-mediated protein transduction is present in both Bacterial and Archaeal domains, and classical endocytosis and macropinocytosis can be used for uncomplexed protein delivery and CPP-mediated protein transduction in cyanobacteria, respectively. Our present results and the recent demonstration of endocytosis-like macromolecular uptake in the planctomycetes (Lonhienne et al. 2010; Fuerst and Sagulenko 2014) are significant advances in our appreciation of both the delivery routes of exogenous cargoes and their potential implications for evolution of macromolecular trafficking in eukaryotic cells.

Conclusions

In this report, we demonstrate that CPPs are able to deliver noncovalently associated proteins into all prokaryotes tested, including cyanobacteria, Gram-negative bacteria, Gram-positive bacteria, and archaea. Classical endocytosis and lipid raft-dependent macropinocytosis are used in cyanobacteria to internalize uncomplexed GFP and CPP/GFP complexes, respectively. Macropinocytosis is a major route for CPP-mediated protein transduction of SR9/QD complexes in DH5α, D-50 bacteria, and archaea. Notably, the CPP-mediated delivery system used in this study was not cytotoxic and did not disrupt membrane integrity. This CPP-mediated protein transduction can be an efficient and safe tool for transformation of proteins and nanoparticles in prokaryotes.

Table 1 Examples of target protein transport into cyanobacteria

Reporter protein	Signal peptide	Species	Localization	Reference
Chloramphenical acetyltransferase	ecolivk rhda sippcy syppcy	*S. elongatus* PCC 7942	Periplasmic space Thylakoid lumen	Mackle and Zilinskas (1994)
Green fluorescent protein	TorA	*Synechocystis* sp. PCC 6803	Periplasmic space	Spence et al. (2003)
Green fluorescent protein	R9	*Synechocystis* sp. PCC 6803 *S. elongatus* PCC 7942	Cytoplasm Cytoplasm, nucleus	Liu et al. (2008)
Organophosphorus hydrolase	Inp	*S. elongatus* PCC 7942	Cell surface	Chungjatupornchai and Fa-aroonsawat (2008)
Green fluorescent protein	InpNC	*S. elongatus* PCC 7942	Periplasmic space	Chungjatupornchai and Fa-aroonsawat (2009)
Organophosphorus hydrolase	SomA	*S. elongatus* PCC 7942	Cell surface	Chungjatupornchai et al. (2011)
Green fluorescent protein	R9	*Synechocystis* sp. PCC 6803 *S. elongatus* PCC 7942	Cytoplasm Cytoplasm, nucleus	Liu et al. (2013a)

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