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Use of bacteria for rapid, pH-neutral, hydrolysis of the model hydrophobic carboxylic acid ester *p*-nitrophenyl picolinate

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Abstract

Caulobacter crescentus, *Escherichia coli* and *Bacillus subtilis* cultures promote the hydrolysis of the model ester *p*-nitrophenyl picolinate (PNPP) at neutral pH with high efficiency. Hydrolysis is related to cell concentration, while the interaction of PNPP with both bacterial cells and their extracellular molecules is required for a maximum rate of PNPP hydrolysis in *C. crescentus* cultures. Furthermore, *C. crescentus* cultures hydrolyze PNPP at concentrations useful in synthetic chemistry.

Keywords

Caulobacter crescentus; green chemistry; hydrolysis; carboxylic esters; *Escherichia coli*; *Bacillus subtilis*

Introduction

Hydrophobic carboxylic acid esters are not soluble in water and require complex methods for hydrolysis, involving surfactants for aqueous solubilization. Bio-mimetic systems including imidazole complexes (Xu et al. 2008), bis-metal chelating ligands (Siqing et al. 2000), and Schiff-base complexes (Hu et al. 2007; Li et al. 2007; Xu et al. 2008) have been used to catalyze the hydrolysis of the model ester *p*-nitrophenyl picolinate (PNPP) in micelles. In an effort to develop more environmentally friendly modes of chemical synthesis, researchers are using biological molecules, such as enzymes, for the preparation of enantiomerically pure chiral synthons (Parales et al. 2002; Testa and Kramer 2007), and yeast to hydrolyze esters (Bialecka-Florjanczyk et al. 2010). Microbial hydroxamates (with the general structure R-CO-NH-OH), which include iron-binding siderophores, can be used as metal chelators. Because hydroxamates are known to be excellent catalysts for the hydrolysis of esters (Menger et al. 2005), we investigated the potential for bacteria to promote hydrolysis of the hydrophobic carboxylic acid ester *p*-nitrophenyl picolinate (Figure 1). PNPP was an ideal choice due to the ease of monitoring hydrolysis by spectrophotometry, and because of its identity as a model ester for hydrolysis studies (Fornasier et al. 1989). Here we show that *C. crescentus*, *B. subtilis* and *E. coli* cultures can all solubilize and hydrolyze PNPP at neutral pH. *Caulobacter crescentus*, a Gram-negative model organism for bacterial cell cycle studies, is ubiquitous in aquatic environments and is

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notable for its ability to survive in very low-nutrient conditions (Ausmees and Jacobs-Wagner 2003). The spore-forming Gram-positive organism *Bacillus subtilis* inhabits the soil whereas *Escherichia coli* is a Gram-negative enteric bacterium.

Materials and methods

Strains and growth conditions

C. crescentus (NA1000), *E. coli* (MG1655), and *B. subtilis* (PY79) were obtained from Lucy Shapiro, Stanford University. *C. crescentus* was grown in 0.02 M HEPES-buffered, pH = 7.0, PYE (Ely 1991) broth (HPYE), *E. coli*, and *B. subtilis* were grown in 0.02 M HEPES-buffered, pH=7.0, Luria Bertani (Davis et al. 1980) broth (HLB) with or without 0.5 mM CaCl₂ and 1 mM MgSO₄ (same concentrations as found in HPYE).

PNPP hydrolysis

We synthesized PNPP (Sigman and Jorgensen 1972) and prepared a fresh working stock solution in acetonitrile for each experiment. A final concentration of 20 or 200 μ M PNPP was achieved by adding 50 μ L aliquots of a PNPP solution, in acetonitrile, of an appropriate concentration to bacterial cultures at various OD_{660 nm} in 15 mm diameter culture tubes at room temperature. PNPP is not soluble enough in acetonitrile to achieve a final concentration of 2 mM without adding a significant volume of acetonitrile to growth media, so solid PNPP was weighed and the appropriate amount added directly to the cultures. Aliquots of the bacterial culture/PNPP solution were removed at several time intervals with a syringe and passed through a 0.22 μ m polyethersulfone (PES) filter to remove the cells. The hydrolysis of PNPP to *p*-nitrophenol and picolinic acid was monitored by measuring the absorbance of the solution in a 100 μ L cuvette (length = 1 cm) at λ = 400 nm (A_{400}) using a Beckman Coulter DU 640 UV-visible spectrophotometer at ambient temperature. Before recording absorbance measurements, the instrument was blanked with bacterial media filtrate without PNPP. The concentration of *p*-nitrophenol in PYE media was calculated using Beer's Law and an experimentally determined extinction coefficient (ϵ) value of 12600 M⁻¹ cm⁻¹, which was obtained by measuring the blank-corrected A_{400} of *p*-nitrophenol standards in 0.02 M HEPES/PYE *Caulobacter* filtrate. Reactions and analysis using *E. coli* (diluted to an OD₆₀₀ of 1.5) and *B. subtilis* (diluted to an OD₆₀₀ of 1.5) were carried out on cultures grown for 24 hours, on rotating platform, at 37°C to an OD₆₀₀ of 3.75 $\pm$ 0.25 for *E. coli* or 8.0 $\pm$ 0.50 for *B. subtilis*. Percent conversions were calculated using experimentally determined ϵ values of 5700 M⁻¹ cm⁻¹ for *E. coli*, and 9800 M⁻¹ cm⁻¹ for *B. subtilis*, obtained by measuring A_{400} of *p*-nitrophenol standards in 0.02 M HEPES/LB *E. coli* MG1655 or *B. subtilis* PY79 filtrate.

Hydrolysis using fractionated culture components

To determine what fraction of the culture had catalytic activity, we assayed supernatants of overnight cultures of *C. crescentus* grown in HPYE for PNPP hydrolysis. To this end, cells were separated from the medium by centrifugation at 5180 $\times g$. The supernatant was isolated and the pellet resuspended in an equal volume of fresh media. To test if centrifugation damaged the extracellular bioconversion factor, *C. crescentus* culture supernatants and cell pellets were separated by centrifugation and the cells then were resuspended in the same supernatant (recapitulated culture). Reaction and analysis were carried out in the same manner described above.

Results and discussion

The purpose of this research was to assess the ability of bacterial species to hydrolyze the model ester *p*-nitrophenyl picolinate (PNPP). When 20 μ M PNPP was added to *Caulobacter*

Crescentus cultures, they immediately turned bright yellow, indicating that *C. crescentus* was able to both solubilize and hydrolyze PNPP, which normally remains insoluble and stable in water or HPYE broth. At an OD_{660 nm} of 1.5 in 15 mm diameter culture tubes, the reaction reached 90% conversion within 5 minutes (Figure 2). When PNPP is added to uncultivated medium, only 10% conversion occurred after 6 hours (not shown). Furthermore, PNPP hydrolysis is dependent on *C. crescentus* cell concentration (Figure 2).

To determine if PNPP hydrolysis was unique to *C. crescentus* cultures, we measured PNPP hydrolysis in *B. subtilis* PY79 and *E. coli* MG1655 cultures and found that both hydrolyze PNPP, albeit less efficiently than *C. crescentus* (Figure 3). Significantly, these results suggest that outer membrane-specific molecules, such as lipopolysaccharide or porins, are not required for PNPP hydrolysis (*B. subtilis* lacks an outer membrane). Furthermore, cultures grown in HLB broth lacking supplemental MgSO₄ and CaCl₂ yielded similar hydrolysis rates (not shown) suggesting neither divalent cation plays a direct role in PNPP hydrolysis. *C. crescentus* was selected for further analysis since it had the highest PNPP hydrolysis rates.

To investigate whether extracellular factors are responsible for PNPP hydrolysis in *C. crescentus*, we fractionated overnight cultures grown in HPYE broth and assayed for PNPP hydrolysis. The supernatant alone was found to promote 40% PNPP hydrolysis relative to the original culture (before centrifugation), whereas the resuspended cell pellet was only able to convert 8% of the amount hydrolyzed by the original culture after 6 minutes (Figure 4). After 30 minutes, the supernatant achieved a total level of hydrolysis similar to that of whole cultures whereas cells only achieved 9% of the hydrolysis (Figure 4). The lower PNPP hydrolysis rates of the supernatants and cells compared to the original cultures suggested that either (1) centrifugation damaged the biocatalytic agent or (2) extracellular molecules require interaction with bacterial cells to achieve maximum PNPP hydrolysis rates. To address this question, supernatants and cell pellets were recombined after centrifugation and PNPP hydrolysis was measured. The recapitulated cultures produced similar PNPP hydrolysis levels as whole cultures (Figure 4). In sum, these results suggest an interaction between the cells and a secreted factor, prompting us to further characterize the extracellular factor. We found that boiling the supernatant eliminated its hydrolytic activity, initially suggesting the existence of an extracellular protein. To test this hypothesis, we incubated the supernatant for 24 hours with 0.5–10 mg/mL trypsin or 75 µg/mL Proteinase K and found that the hydrolytic activity was neither trypsin- nor Proteinase K-sensitive (not shown), suggesting that the extracellular molecule is not proteinaceous. We confirmed that trypsin and Proteinase K were effective proteolytic agents on BSA (not shown) in HPYE broth as well as in buffered *C. crescentus* filtrate (OD_{660 nm} = 1.5). Thus, the secreted PNPP hydrolysis-promoting factor is a protease-resistant heat-labile molecule, which retains activity after 24 hours at 37°C (not shown).

To determine if *C. crescentus* could hydrolyze concentrations of PNPP that would be useful in synthetic chemistry, 200 µM or 2 mM PNPP was added to *C. crescentus* cultures and hydrolysis was monitored for two hours. Figure 5 shows that *C. crescentus* efficiently hydrolyzes PNPP concentrations of 200 µM and 2 mM, concentrations useful in synthetic chemistry, achieving 84% and 52% conversion respectively after 120 minutes. Additionally, preliminary results suggest that *C. crescentus* can be used to hydrolyze other hydrophobic carboxylic esters that may be of use to synthetic chemists including pivalate and isobutyrate esters (not shown). These esters are employed as protecting groups for alcohols, but their removal requires relatively harsh conditions due to their steric bulk. For example, diisobutylaluminum hydride is necessary for cleaving pivalates (Carreira and Du Bois 1995; Nicolaou and Webber 1986). This method gives high yields but is incompatible with alkenes, alkynes and ketones because it reduces these functional groups. Similarly, sodium

hydroxide in ethanol or isopropanol is used to hydrolyze pivalate and isobutyrate esters (Barnes et al. 2006; Ogilvie and Iwacha 1973). However, these basic conditions are incompatible with certain substrates such as methyl-(S)-2-methyl butyrate which is susceptible to racemization, and ethyl β -hydroxyhydrocinnamate which is prone to dehydration.

We have developed a novel and environmentally friendly approach to hydrolyzing the model ester *p*-nitrophenyl picolinate using *Caulobacter* in aqueous solution at pH 7.0. We believe that this approach may become useful to synthetic chemists looking to hydrolyze ester molecules that are not compatible with traditional chemical hydrolysis methods.

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Figure 1.
p-nitrophenyl picolinate (PNPP) can be hydrolyzed to picolinic acid and *p*-nitrophenol, a chromogenic molecule.

Figure 2. PNPP hydrolysis by *C. crescentus* is related to cell concentration. The rate of hydrolysis of 20 μ M PNPP increases with cell concentration of *C. crescentus*. Averages of triplicate experiments are shown with y-error bars.

Figure 3. *B. subtilis* and *E. coli* hydrolyze PNPP, albeit less efficiently than *C. crescentus*. Hydrolysis of 20 μ M PNPP by *C. crescentus*, *B. subtilis*, and *E. coli* is shown. Averages of triplicate experiments are shown with y-error bars.

Figure 4. Maximum rates of PNPP hydrolysis result from an interaction of bacterial cells with extracellular molecules in *C. crescentus* cultures. A comparison of hydrolysis of 20 μM PNPP by whole cultures, reconstituted cultures, cells only, or supernatant only of *C. crescentus* cultures. Averages of triplicate experiments are shown with y-error bars.

Figure 5.

C. crescentus hydrolyzes PNPP at concentrations useful in synthetic chemistry. Averages of triplicate experiments are shown with y-error bars.