Short communication

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Proteomic changes in *Escherichia coli* TG1 after metabolic engineering for enhanced trichloroethene biodegradation

Through metabolic engineering, new enzymatic pathways can be introduced into cells to enable or enhance production or biotransformation of chemicals. However, these changes have physiological consequences that can be important but are not well understood. Here we describe the use of two-dimensional gel electrophoresis (2-DE) to detect changes in the proteome of *Escherichia coli* cells that have been engineered to transform the pollutant trichloroethene (TCE) with the enzyme toluene *o*-monooxygenase (TOM). Comparison of 2-DE gels (isoelectric point range 4–7) for *E. coli* cells with and without the ability to synthesize TOM revealed 31 new proteins in TOM-containing cells as well as nine proteins not detected in those cells but present in the plasmid control strain. Exposure of TOM-containing cells to TCE led to the synthesis of four new proteins and the loss of only one protein. Thus, this example of metabolic engineering has a substantial and complex impact on the physiology of these cells that was clearly revealed using a proteomic approach.

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Trichloroethene (TCE) is a suspected human carcinogen and one of the most frequently reported groundwater pollutants [1]. Although this contaminant can be biodegraded anaerobically by reductive dechlorination, the proper conditions for complete dechlorination are often not achievable and the accumulation of lesser-chlorinated ethenes, including the more toxic vinyl chloride, can occur [2]. TCE can also be degraded aerobically by cometabolism, which involves the nonspecific action of oxygenases. However, aerobic cometabolism of TCE is problematic to achieve because it requires the addition of an inducer for the responsible enzyme (toluene-*o*-monooxygenase, TOM) and an energy source, and because a cytotoxic intermediate (TCE epoxide) is generated [3, 4]. Metabolic engineering involves genetic manipulations to alter enzymatic, regulatory, and/or nutrient transport activities within cells [5]. This approach offers a means to overcome the shortcomings of aerobic cometabolism by manipulating the promoter to allow uninduced production of TOM, evolving the activity of TOM to achieve higher rates of TCE degradation, and cloning an additional enzyme to react with and protect the cell from toxic intermediates. However, the production of new enzymes and/or changes in a regulatory mechanism can have unforeseen effects on the physiology of the host organism, with possibly detrimental consequences to the desired application [5, 6].

Unfortunately, few analyses of the effects of metabolic engineering have focused on overall changes in host cell physiology as measured by changes in protein production or other cellular processes. Since most cellular processes are either regulated or directly carried out by proteins or protein complexes, physiological responses to new genes can be expected to result in altered production of various host cell proteins other than those introduced in the genetic manipulation [7]. Thus, proteomic analyses of metabolically engineered cells are expected to yield valuable insights into the overall cellular pathways that are affected by metabolic manipulation. Specifically, 2-DE offers an ideal approach to study changes in a subpopulation of a cell's proteome because of its ability to resolve complex protein mixtures into individual polypeptides. The first step of this project involved construction of the *Escherichia coli* TG1 pBS(Kan-)/TOM clone which constitutively expresses the genes for the six subunits of the

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Abbreviations: Kan, Kanomycin; **TCE**, trichloroethene; **TOM**, toluene-*o*-monooxygenase

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TOM enzyme from a high copy number plasmid [8]. In addition, a plasmid control clone (*E. coli* TG1 pBS(Kan-)) was constructed that harbors the plasmid without the *tom* genes. With this clone, the effects of TOM production can be differentiated from those effects specific to plasmid expression. Finally, cells containing these plasmid-borne genes were exposed to TCE so that the same analyses could be applied to the effects of TCE exposure and TCE biotransformation.

Cultures of *E. coli* TG1 pBS(Kan-) and *E. coli* TG1 pBS(Kan-)TOM were started by streaking −80°C glycerol (15% v/v) stocks on Luria Bertani (LB) agar plates with 100 μg/mL kanamycin and incubated at 30°C for 24 h. Individual colonies were then inoculated into 5 mL seed cultures of chloride-free M9 medium [9] (amended with maltose (0.4% w/v), $FeSO_4$ (1.0 mg/L), and kanamycin (100 μg/mL)) and incubated at 30°C and 200 rpm to an OD (600 nm) of 0.4 to 0.8 (mid-exponential phase). One milliliter of each seed culture was used to inoculate 100 mL of the same medium in a 500 mL Erlenmeyer flask and incubated at 30°C and 200 rpm to mid-exponential phase. If applicable, cells were exposed to TCE *via* the vapor phase by adding 100 μL of solvent to a small tube suspended from a rubber stopper in the top of the flask when the culture had reached an OD (600 nm) corresponding to a point approximately two hours prior to mid-exponential phase, then continuing the incubation for two hours.

Cells were harvested by centrifugation at 10 000 × *g* and 15°C for 20 min. Cell pellets were subsequently washed two times as described by Hatzimanikatis *et al.* [10]. The final washed pellet was suspended in a sonication buffer [10] containing 10 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% w/v SDS, 0.5 mM DTT, and 0.5 mM Pefabloc SC (Boehringer, Indianapolis, IN, USA), and sonicated on ice at maximum power (5 min total on time; 1 s on: 2 s off) in a Fisher (Houston, TX, USA) 550 Sonic Dismembrator equipped with a microtip. The lysate was then centrifuged at 11 300 × *g* and 15°C for 20 min to remove cell debris, and the supernatant was aliquoted and stored at −80°C until use. The protein concentration of each lysate supernatant was determined using the Bradford-based protein assay from Bio-Rad (Hercules, CA, USA).

IEF was carried out using 18 cm, pH 4–7 linear Immobiline IPG gels (Amersham Biosciences, Piscataway, NJ, USA). The sample was loaded by in-gel rehydration by mixing 100 μg of protein sample with reswelling solution [10] containing 8 M urea, 2.0% w/v CHAPS, 0.3% w/v DTT, 2.0% v/v pH 4–7 IPG buffer (Amersham Biosciences), and a trace of bromophenol blue, to a final volume of 400 μL. This final sample mixture was applied to an IPG gel, which was incubated at room temperature for 10 h. IEF was carried out for 76 500 Vh at 20°C in a Multiphor II (Amersham Biosciences), wherein the voltage was linearly increased from 500 V to 3500 V over the first 5 h, and then maintained at 3500 V for the final 17.5 h by an EPS 3500 XL power supply (Amersham Biosciences). Equilibration was carried out as described by Hatzimanikatis *et al.* [10]. After equilibration, the IPG gels were transferred to the top of 12% SDS slab gels (18 cm × 16 cm × 1.0 mm), with the IPG gels pressed firmly to the slab gel surface to ensure successful protein transfer. SDS-PAGE was carried out in a Protean II xi Multi-Cell (Bio-Rad) at 40 mA *per* gel, using the EPS 3500 XL power supply, until the bromophenol blue front had migrated to within a few millimeters of the bottom of the slab gel. Proteins were detected using the PlusOne Silver Staining Kit (Amersham Biosciences), without the addition of glutaraldehyde. Gel images were analyzed using the PDQuest 2-D analysis software (Bio-Rad). Duplicate gels of each type were analyzed.

The observed growth curves of the two *E. coli* TG1 clones showed that the plasmid control variant grew to approximately the same extent as the pBS(Kan-)TOM variant. The yield of *E. coli* TG1 pBS(Kan-) was approximately 0.33 mg dry cell mass/mg maltose, compared to a yield of about 0.30 mg dry cell mass/mg maltose for *E. coli* TG1 pBS(Kan-)TOM. The slightly lower yield achieved by *E. coli* TG1 pBS(Kan-)TOM is expected due to the higher catabolic burden on this variant to express the six subunits of the TOM enzyme from a high copy number plasmid, as well as the depletion of energy stores within the cell due to the activity of the TOM enzyme within the cell (which requires NADH as a cofactor).

A comparison of the proteomic profiles of *E. coli* TG1 pBS(Kan-) and *E. coli* TG1 pBS(Kan-)TOM is shown in Fig. 1. Analysis of the proteomes obtained for these variants revealed that eight proteins were expressed by the plasmid control clone (Fig. 1A) but were present below detection limits in the clone expressing the *tom* genes, while 30 proteins were observed only in the pBS(Kan-)-TOM clone (Fig. 1B). Although there are six genes encoding for the six individual subunits of the TOM enzyme, only five of these subunits are expected in the presented gel image shown in Fig. 1B, since the theoretical p*I* of one subunit (A_4, p*I* 7.35) is beyond the pH range covered by the IPG gels used in this study (pH 4–7). Therefore, up to five of the 30 proteins observed only in the proteome of *E. coli* TG1 pBS(Kan-)TOM could be expected to correspond to the remaining subunits. The theoretical p*I* values and M_r of the five subunits were compared to the approximate p*I* values and M_r observed for the 30 spots of interest in the *E. coli* TG1 pBS(Kan-)TOM proteome to determine if any preliminary matches could be made. All five

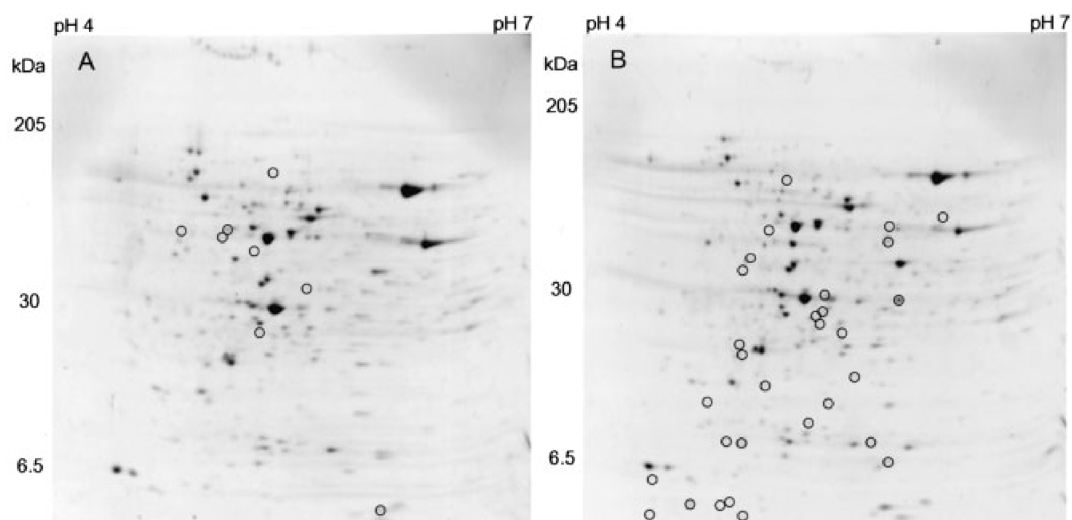

Figure 1. 2-DE gel images for (A) *E. coli* TG1 pBS(Kan-) and (B) *E. coli* TG1 pBS(Kan-)TOM. Circles enclose spots that are produced only in the proteome of the specified clone of each image.

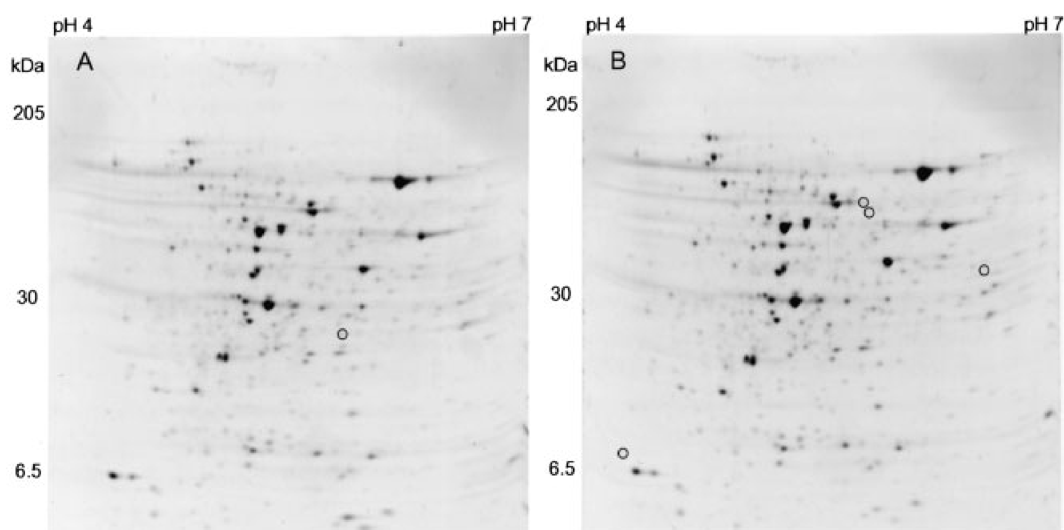

Figure 2. 2-DE gel images for (A) *E. coli* TG1 pBS(Kan-)TOM without TCE exposure and (B) *E. coli* TG1 pBS(Kan-)TOM with TCE exposure. Circles enclose spots that are produced only in the proteome of *E. coli* TG1 pBS(Kan-)TOM grown under the specified conditions of each image.

subunits were tentatively matched to five of the dynamic spots for this proteome. Identification of these spots by mass spectrometry is in progress to confirm these tentative matches.

The proteomic profiles of *E. coli* TG1 pBS(Kan-)TOM with and without exposure to TCE are shown in Fig. 2. Analysis of these proteomes revealed that four new proteins were produced in response to TCE biotransformation by TOM-expressing cells exposed to TCE (Fig. 2B). However, TCE biotransformation led to an apparent repression in the synthesis of one protein that was expressed by the same strain in the absence of TCE (Fig. 2A). The four proteins newly expressed in the presence of TCE may be involved in DNA or other cellular repair mechanisms [11] induced in response to damage caused in this clone by TCE epoxide [3, 4], a reactive intermediate arising from the reaction of TOM with TCE in the medium. The one protein that was detected in this clone only when TCE is not present in the medium may be involved in mechanisms that are only induced when the cell is actively growing. In this way, addition of TCE to the medium, and the subsequent production of TCE epoxide by TOM, would require the cells to devote energy and carbon resources to repairing

the damage inflicted by the epoxide and to repress pathways and enzymes whose expression is not critical to such repair or maintenance pathways. Identification of these differentially synthesized proteins is underway to evaluate these hypotheses. A summary of both proteomic analyses is presented in Table 1.

Table 1. Summary of 2-DE analyses of *E. coli* TG1 proteomes for duplicate gels

Effect	Gel comparison	New proteins	Absent proteins[a)]
Synthesis of TOM	pBS(Kan-) *vs.* pBS(Kan-)TOM (Fig. 1)	30	8
TCE biotransformation	pBS(Kan-)TOM with and without TCE (Fig. 2)	4	1

a) Below detection limit for this silver staining protocol (approx. 0.2 ng *per* spot).

In conclusion, proteomic analysis of the protein profiles of two clones, as well as the analysis of the proteomes of these clones under different environmental conditions, revealed distinct changes in the expression levels of multiple proteins. These results support the use of proteomics to monitor and investigate more subtle physiological consequences of metabolic engineering. Once the proteins of interest are identified, a more precise description of the specific pathways and mechanisms invoked in response to metabolic engineering can be developed, and optimization of the metabolic engineering approach can be pursued.

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