

## Proteome Analysis of Acrylamide-induced Proteins in a Novel Acrylamide-degrader *Enterobacter aerogenes* by 2D Electrophoresis and MALDI-TOF-MS

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### ABSTRACT

Despite tremendous advances in understanding the microbial degradation of acrylamide, reports on the nature of the two-dimensional protein patterns for acrylamide-degrading bacteria are not yet available. This work, focusing on the acrylamide-inducible proteins, studied the response of *Enterobacter aerogenes*, a novel acrylamide-degrading bacterium, to acrylamide. Proteome analysis was applied using 2D-polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionisation-time of flight mass spectrometry to identify proteins differentially expressed from *E. aerogenes* grown on acrylamide. Six protein homologues with amidohydrolase, urease accessory protein, quaternary ammonium compound resistance proteins, dipeptide transport protein, Omp36 osmoporin and large conductance mechanosensitive channel proteins (MscL) are seemingly involved in acrylamide stress response and its degradation. Five proteins identified as GroEL-like chaperonin, ArsR-transcriptional regulator, Ts- and Tu-elongation factor and trigger factor and four proteins (phosphoglycerate kinase, ATP synthase  $\beta$ -subunit, malate dehydrogenase and succinyl-CoA synthetase  $\alpha$ -subunit) responsive for the adaption of *E. aerogenes* in the presence of acrylamide

**Keywords:** Acrylamide, Biodegradation, *Enterobacter aerogenes*, 2D-PAGE, MALDI-TOF MS

### INTRODUCTION

Acrylamide ( $\text{CH}_2=\text{CHCONH}_2$ ) is used as a conjugated reactive molecule in polyacrylamide production as well as a binding, thickening or flocculating agent

in industrial applications (Prasad, 1982; Wampler and Ensign, 2005; Prabu and Thatheyus, 2007). Demand for acrylamide increases with industrial and domestic applications for polyacrylamides and other polymers (Prasad, 1982; Nagasawa and Yamada, 1989; Wang et al., 2009). However, acrylamide is highly neurotoxic, carcinogenic and teratogenic in animals and considered an environmental contaminant (Cherry et al., 1956; Croll et al., 1974; Tilson and Cabe, 1979; IARC, 1994; Segerbäck et al., 1995; Prabu and Thatheyus, 2007). Bioremediation of acrylamide to non-harmful substances would alleviate environmental concerns.

Microbial degradation of acrylamide has been explored extensively with a diversity of isolates, such as *Bacillus*, *Pseudomonas* and *Rhodococcus* (Yamada et al., 1979; Thiery et al., 1986; Nawaz et al., 1993; Hirrlinger et al., 1996; Wang and Lee, 2001; Shukor et al., 2009a, b). Acrylamide is initially deamidated to ammonia and acrylic acid, a process catalyzed by amidase or amidohydrolase (EC 3.5.1.4) (Shanker et al., 1990; Nawaz et al., 1994; Nawaz et al., 1998; Zabaznaya et al., 1998). Wampler and Ensign (2005) proposed acrylic acid is degraded initially to  $\beta$ -hydroxypropionate, then oxidized to CO<sub>2</sub> or reduced to propionate.

In a previous study, a novel acrylamide-degrading bacterium, *Enterobacter aerogenes* was isolated and characterized from domestic wastewater in Chonburi, Thailand. The strain grew well in acrylamide as 0.5% (w/v), at pH 6.0 to 9.0 and 25°C, that was degraded to acrylic acid in the late logarithmic growth phase in a biomass-dependent pattern. Activity in cell-free supernatant was sufficient to completely degrade butyramide and urea, and other aliphatic amides to acrylic acid (Buranasilp and Charoenpanich, 2011). However, it remains unknown how acrylamide enter bacteria and the degradation process after its early conversion to acrylic acid. Other proteins may be involved in acrylamide and acrylic acid degradation. Cellular proteins can be separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (Klose, 1975; O'Farrell, 1975) and their apparent molecular weight and quantity measured allowing identification and is a powerful tool to study changes in cellular protein expression (Choe and Lee, 2000). The objective of the present study was to document the entire protein complement induced by acrylamide, using a combination of 2D-PAGE and MALDI-TOF mass spectrometry.

## MATERIALS AND METHODS

### Cell cultivation and harvesting

*E. aerogenes* (Buranasilp and Charoenpanich, 2011) was cultivated at 25°C in W-minimum medium (Kimbara et al., 1989) that contained 0.5% (w/v) acrylamide or glucose until the late exponential phase (OD<sup>600</sup> ~ 1.0). Cells were harvested by centrifugation at 5,000 ×g for 10 min at 4°C, washed with 0.85% (w/v) NaCl, and stored at -80°C until use.

### Preparation of protein fraction

Cell pellets were resuspended in 5-ml of lysis buffer I (10 mM Tris-HCl, 1 mM EDTA at pH 8.0, 1 mM PMSF and 1 mM DTT) and disrupted by ultrasonica-

tion (Buranasilp and Charoenpanich, 2011). Nuclease solution (1 mg/ml DNaseI, 0.25 mg/ml RNaseA, 50 mmol/l MgCl<sub>2</sub>, 24 mM Tris (base) and 476 mM Tris-HCl) at 1/10 volume was added and the mixture incubated on ice (<4°C) for 20 min to remove nucleic acid contaminants. Intact cells were removed by centrifugation at 10,000 ×g for 30 min, <4°C. The supernatant was collected by centrifugation at 40,000 ×g for 45 min (4°C), precipitated in 9 volumes of acetone solution I (10% (w/v) TCA and 0.3% (w/v) DTT in acetone) and kept overnight at -20°C. Proteins in the precipitate were collected by centrifugation at 5000 ×g for 5 min and washed with an appropriate volume of acetone solution II (0.3% (w/v) DTT in acetone). Washed proteins were then dissolved for 30 min in solubilization buffer (8 mM urea, 4% (w/v) CHAPS, 60 mM DTT, 2% (v/v) Bio-Lyte pH 3-10 (Bio-Rad, USA), and 0.002% (w/v) bromophenol blue). Insoluble components and reagent contaminants were removed by centrifugation at 15,000 ×g for 15 min. Dissolved proteins were determined spectrophotometrically (Bradford, 1976) using Bio-Rad assay reagent (Hercules, USA) and bovine serum albumin as the standard.

### **Two-dimensional electrophoresis**

An immobilized gel strip (18 cm) containing a pH gradient of 5-8 (IPG) (Bio-Rad, USA) was rehydrated at 50 V, 20°C for 12 h with 350 µl of rehydration solution (8M urea, 0.5% (w/v) CHAPS, 0.2% (w/v) DTT, 0.5% (v/v) Bio-Lytes pH 3-10, and 0.002% (w/v) bromophenol blue) that contained 1 µg of protein sample. IEF was conducted at 20°C as follows: S1 – rapid voltage at 100 V for 1 h; S2 – rapid voltage at 500 V for 1 h; S3 – rapid voltage at 10,000 V for 10 h; S4 – rapid voltage from 10,000 to 60,000 V for 16 h; and S5 – rapid voltage at 500 V for 20 min. The limit voltage was 50 µA/gel strip. After focusing, gel strips were equilibrated first in solution I (50 mM Tris-HCl at pH 8.8, 6M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 2% (w/v) DTT, and 0.002% (w/v) bromophenol blue), followed by solution II (50 mM Tris-HCl at pH 8.8, 6M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 2.5% (w/v) iodoacetamide, and 0.002% (w/v) bromophenol blue) with gentle shaking for 10 min. Then, the IPG was embedded in a discontinuous SDS-PAGE gel (Laemmli, 1970) (15% separating gel, 5% stacking gel). Electrophoresis buffer consisted of 25 mM Tris-HCl at pH 8.3, 192 mM glycine, and 0.1% (w/v) SDS. Gels were run at constant current as 20 mA per gel until bromophenol blue moved into the separating gel, and then the current was raised to 50 mA per gel until complete. The gels were fixed in prefixative solution (20% (v/v) methanol and 7.5% (v/v) acetic acid) for 30 min, stained (50% (v/v) methanol, 10% (v/v) acetic acid, and 0.05% Coomassie brilliant blue R-250) for 45 min, and destained (5% (v/v) methanol and 7% (v/v) acetic acid) until the gel background was clear.

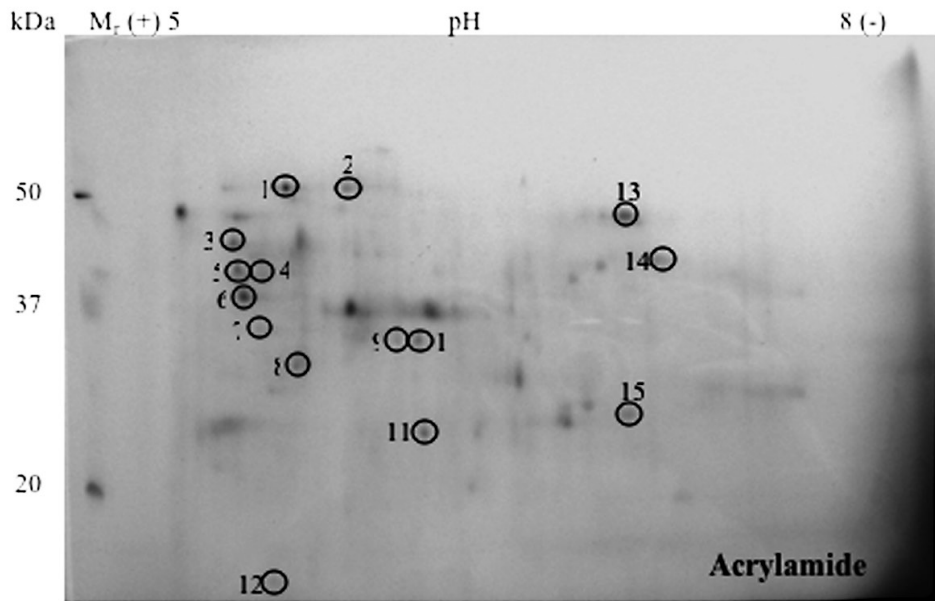
### **In-gel digestion and MALDI-TOF MS analysis**

Protein spots from cells grown in acrylamide and glucose were compared, excised from 2-D gels and digested in trypsin (MS-grade, Sigma, USA) according to the proteomic protocols for mass spectrometry (Bruker Daltonics). Samples were

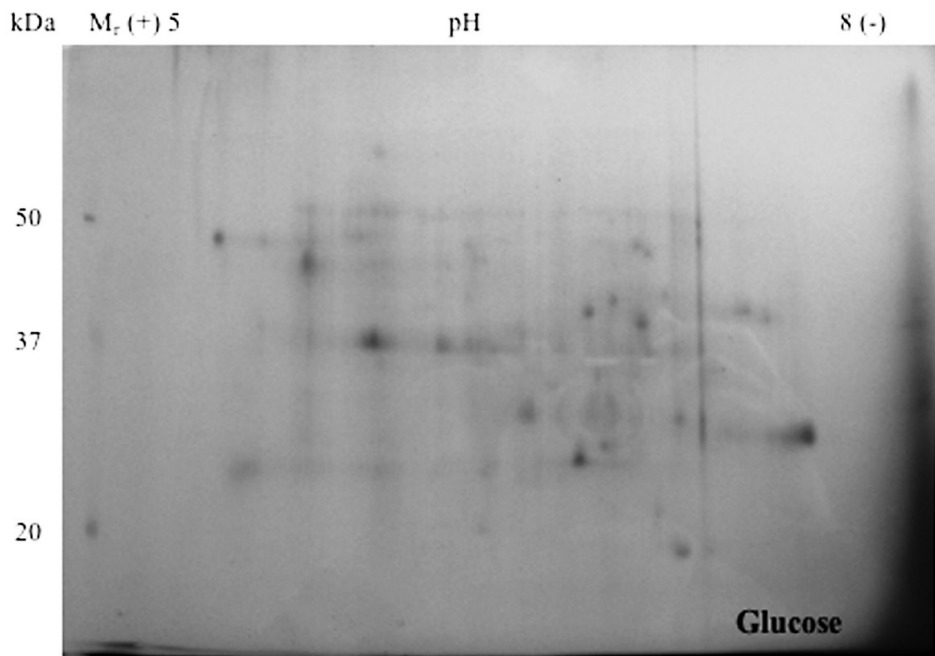
concentrated and purified by ZipTipC18 resin (Millipore, England). Samples (0.8  $\mu$ l) were then spotted on an AnchorChip target (Bruker, Germany) and analyzed with an UltraFLEX MALDI-TOF mass spectrometer (Bruker, Germany). Calibration was done with a peptide calibration standard, molecular mass 1 to 4 kDa (Bruker, Germany). The matrix solution was 0.3 mg/ml  $\alpha$ -cyano-4-hydroxycinnamic acid (Bruker, Germany) in 50% acetone in ethanol. Trypsin contamination peak was excluded from the database peak list. Each spectrum was produced by accumulating data from 200 consecutive laser shots. Parent masses were analyzed with Biotoools version 2.2 (Bruker, Germany) and amino acid sequences were identified using homology searches in the MS database (<http://dove.embl-heidelberg.de/Blast2/msblast.html>). Search parameters were as follows: type of search – peptide mass fingerprint; enzyme – trypsin; fixed modification – carbamido methylation (Cys); variable modifications – oxidation (Met); mass values – monoisotopic; peptide charge state – 1+; maximum missed cleavages – 1; and peptide mass tolerance – 0.05% Da (50 ppm). Statistical analyses of the sequences were determined by the probability-based Mowse score offered by the software. A *p*-value of < 0.05 was considered significant and used to generate the results.

## RESULTS

In media from acrylamide-grown bacteria, 15 proteins were separated on 2D-gel run with a pH gradient of 5 to 8 with molecular sizes of approximately 56 (pI 5.7), 59 (pI 5.9), 41 (pI 5.3), 42 (pI 5.5), 60 (pI 5.4), 46 (pI 5.4), 15 (pI 5.5), 25 (pI 5.8), 11 (pI 6.2), 30 (pI 6.3), 33 (pI 6.3), 12 (pI 5.6), 48 (pI 7.1), 43 (pI 7.2), and 30 (pI 7.1) respectively (Fig. 1). Spots were cut from gels and subsequently digested in trypsin. Consequently, the digestate was analyzed by MALDI-TOF MS to determine molecular masses of the tryptic peptides. Sequences were compared with those in databases and potential identities are summarized in Table 1.



(a)



(b)

**Figure 1.** Proteins in (a) acrylamide- (b) glucose-grown *E. aerogenes* separated by 2D-gel electrophoresis using pH 5-8 IPG and 15% SDS-PAGE. Proteins were stained with Coomassie brilliant blue R-250. Different protein spots are circled and the identified proteins are labeled in Table 1.

**Table 1.** Summary of proteins identified from the 2D-gel of acrylamide-induced protein in *E. aerogenes*, compared with the cells grown on glucose and acrylamide.

Spot #*	Calculated masses	kDa	Expected protein	Source	Accession no. in database**
1	ANDAAGDGTTTATVLAQAIVNEGLK	56	GroEL-like chaperonin	<i>E. aerogenes</i>	gi 165974054
2	TLVYCSEGSPEGFNPQLFTSGTTY DASSVPIYNR	59	Dipeptide transport protein	<i>Klebsiella pneumoniae subsp. pneumoniae</i> MGH 78578	gi 152972407
3	FADVACAGPLLAEELDALGK	41	Phosphoglycerate kinase	<i>E. cancerogenus</i> ATCC 35316	gi 261342323
4	IDGLHYFSSDDSDVDGQTYMR	42	Omp36 osmoporin	<i>E. aerogenes</i>	gi 13384121
5	ILAVGGPAEMAPWPDAEPVDTR	60	Amidohydrolase	<i>Ralstonia eutropha</i> JMP 134	gi 73539577
6	MPSAVGYQPTLAEEMGVLQER	46	ATP synthase $\beta$ -subunit	<i>E. aerogenes</i>	gi 112791340
7	QFAFTLR	15	Large conductance mechanosensitive channel proteins	<i>E. cloacae subsp. cloacae</i> NCTC 9394	gi 295096934
8	ACEQGDIAAAQRWTAYLLACR	25	Urease accessory protein	<i>K. aerogenes</i>	UREF_KLE-AE
9	MIPNHPEPEQIQLENVLFALGNPLR	11	Transcriptional regulator ArsR	<i>E. cancerogenus</i> ATCC 35316	gi 261339903
10	ALTEANGDIELAIENMR	30	Elongation factor Ts	<i>K. pneumoniae subsp. pneumoniae</i> MGH 78578	gi 152968764
11	IKGFSGEDATPALEGADVVLISAG VAR	33	Malate dehydrogenase	<i>K. pneumoniae subsp.</i>	gi 152972158
12	KNWIFLAVSIFGEVIATSALK	12	Quaternary ammonium compound	<i>E. aerogenes</i>	QACF_EN-TAE
13	AGEEFTIDVTFPEEYHAENLK	48	Trigger factor	<i>E. cloacae subsp. cloacae</i> NCTC 9394	gi 295096777
14	AIDKPFLLPIEDVFSISGR	43	Elongation factor Tu	<i>K. pneumoniae subsp. pneumoniae</i> MGH 78578	gi 152972232
15	EHVTKPVVGYIAGVTAPK	30	Succinyl-CoA synthetase $\alpha$ -subunit	<i>E. cloacae subsp. cloacae</i> ATCC 13047	gi 296103342

Note: \*The spot numbers listed in the first column correspond to the spot numbers in the 2D-gel in Fig. 1.  
 \*\*MS database (<http://dove/embl-heidelberg.de/Blast2/msblast.html>).

Enzymes involved in energy metabolism are represented by four protein spots (phosphoglycerate kinase [Spot #3], ATP synthase  $\beta$ -subunit [Spot #6], malate dehydrogenase [Spot #11], succinyl CoA synthetase  $\alpha$ -subunit [Spot #15]) and five, in the synthesis and transport of proteins (GroEL-like chaperonin [Spot #1], transcription regulator ArsR [Spot #9], elongation factor Ts [Spot #10] and Tu [Spot #14], trigger factor [Spot #13]). The acrylamide stress response may also involve the gel-abundant dipeptide transport proteins [Spot #2], Omp 36 osmoporin [Spot #4], amidohydrolase [Spot #5], large conductance mechanosensitive channel protein [MscL, Spot #7], urease accessory protein [Spot #8] and quaternary ammonium compound resistance protein [Spot #12].



## DISCUSSION

Until now, we cannot deny possible routes for acrylamide other than deamination via amidase (Nawaz et al., 1994; Hirrlinger et al., 1996; Cha & Chambliss, 2011). The subsequent fate of acrylate is not well understood, but probably involves pathways and enzymes that have been characterized to various degrees for other acrylate-utilizing bacteria. Acrylate metabolism is believed to proceed via hydroxylation to  $\beta$ -hydroxypropionate, then oxidized to  $\text{CO}_2$  (Shanker et al., 1990) or reduced to propionate (Wampler and Ensign, 2005). Another plausible pathway for mineralization of acrylamide is via formation of acrylyl CoA, which eliminates lactate as a final product (Shanker et al., 1990).

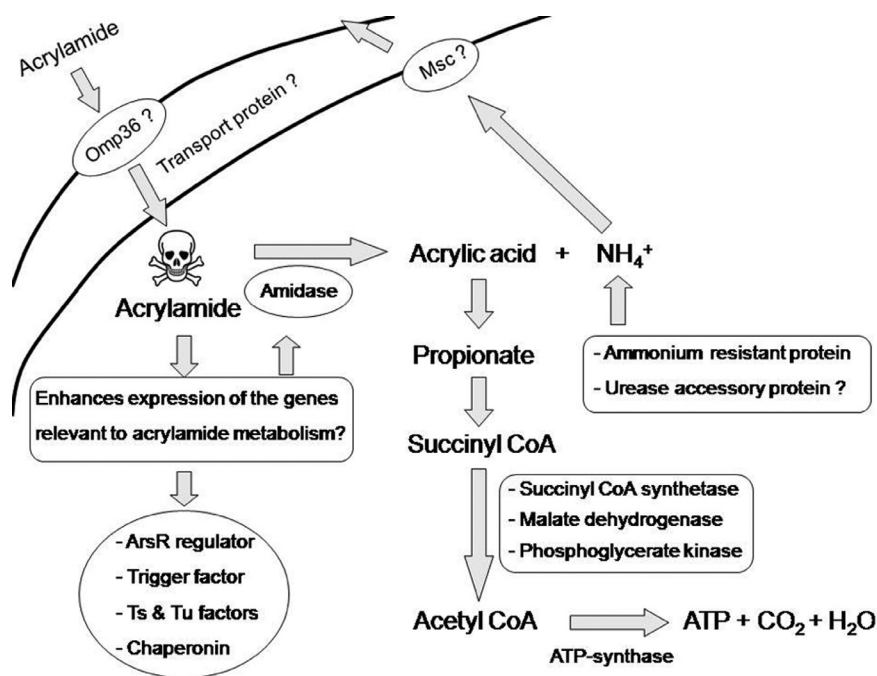
A powerful tool that also enables unraveling acrylamide metabolic pathways is the sequential induction of catabolic enzymes and intermediary metabolites. Further, insight into degradative pathways is also provided from assaying the probable key proteins that are synthesized at sufficient levels when acrylamide is present. Using proteome analysis, fifteen proteins differentially expressed from *Enterobacter aerogenes* grown on acrylamide were identified. Five proteins identified as GroEL-like chaperonin, ArsR-transcriptional regulator, Ts- and Tu-elongation factor and trigger factor and four proteins (phosphoglycerate kinase, ATP synthase  $\beta$ -subunit, malate dehydrogenase and succinyl-CoA synthetase  $\alpha$ -subunit) are expected to be relevant to adaption of *E. aerogenes* in the presence of acrylamide. The metabolic changes may be a consequence of the biological cost of acrylamide resistance, although other forms of these proteins may exist and are not resolved on these 2D-gels. Although chaperonin provides essential kinetic assistance to protein folding, expression of this protein for cell survival in nutrient limitations have also been documented (Fayet et al., 1989; Fischer et al., 1993). Chaperonin may be required for stress-induced survival in acrylamide. ArsR transcription regulator implicitly represents a detoxification mechanism that has endowed prokaryotes to respond to externally induced stress. Most appear to be negative regulators, and many derepress by direct binding of metal ions (Busenlehner et al., 2003). Thus, and perhaps most interesting, this regulator might be necessary for expression of genes relevant in acrylamide metabolism. However, the functions of this regulator in acrylamide degradation are to be elucidated. Elongation factor Ts and Tu are expected to be the essential proteins for transcription and translation of proteins involved in acrylamide degradation.

Six protein homologues with amidohydrolase, urease accessory protein, quaternary ammonium compound resistance proteins, dipeptide transport protein, Omp36 osmoporin and large conductance mechanosensitive channel proteins (MscL) are seemingly involved in acrylamide stress response and its degradation. The outer membrane of gram-negative bacterial cells forms a protective barrier against damaging external agents. Porins in the membrane form hydrophilic channels, allowing selective uptake of essential nutrients (Koebnik et al., 2000; Nikaido, 2003). Synthesis of porins may be up- or down-regulated by the presence or absence of molecules in the medium or inducer threshold concentrations (Barbosa and Levy, 2000; Pomposiello et al., 2001; Nikaido, 2003). Perhaps *E. aerogenes* uses Omp36 osmoporin to assimilate acrylamide. Systems to trans-

port peptides occur in many species and generally function to accumulate intact peptides intracellularly, where they are hydrolyzed (Payne, 1980). The dipeptide transport protein, one of the osmotic-shockable transport systems of gram-negative bacteria is responsible for the uptake of many types of small molecules (Payne, 1980; Payne and Smith, 1994). As an amide, the movement of acrylamide from periplasm into the cell of *E. aerogenes* might be facilitated by dipeptide transport protein

The present study confirms the production of amidohydrolase (amidase) by *E. aerogenes* during acrylamide degradation and is in accord with earlier reports (Shanker et al., 1990; Nawaz et al., 1994; Nawaz et al., 1998; Buranasilp and Charoenpanich, 2011; Thanyacharoen et al., 2012). MscL protein of the mechanosensitive channel with large conductance appears to play an important role in the integrity of cell membranes by regulating the flow of ions and other small solutes (Ajouz et al., 1998; Berrier et al., 2000). Thus, MscL might release ammonium or other ions from *E. aerogenes* for cell stabilization. This hypothesis is confirmed by the release of ammonium ion into the media of *E. aerogenes* (Buranasilp and Charoenpanich, 2011). The functions of quaternary ammonium compound resistance protein as well as urease are still ambiguous but may be involved in ammonium detoxification.

Although enzyme assays and other physiological data on *E. aerogenes* are still needed, the present study suggests acrylamide may be assimilated using Omp36 osmoporin and dipeptide transport proteins (Figure 2).



**Figure 2.** Model of acrylamide uptake and the subsequent fate of acrylamide possible occurred in *E. aerogenes*. Details are given in the text.



Acrylamide is toxic, indeed lethal, to most microorganisms. However some bacteria, including *E. aerogenes*, have adapted their metabolism to use this substance as an energy source. Important to this adaptation is the evolution of genes that encode amidohydrolase (amidase) and other synthesis proteins that deaminate acrylamide to acrylic acid and ammonium (Shanker et al., 1990; Nawaz et al., 1994; Nawaz et al., 1998; Buranasilp and Charoenpanich, 2011; Thanyacharoen et al., 2012). With this, acrylic acid can be changed to propionate and subsequently succinyl CoA (Stams et al., 1993; Ansedo et al., 1999; Wampler and Ensign, 2005) to generate energy. Potentially harmful ammonium is detoxified and MscL protein is released from the cell.

Classical proteomic approaches mostly provide information on relative amounts of protein species and only rarely information on activity. Precise functional understanding of the biological systems requires metabolomics and interaction studies. In conclusion, this paper provides important missing information on bacterial responses to acrylamide. Further transcriptional and translational studies would lead to the total resolution of the acrylamide degradation.

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