Characterization of eukaryotic-like kinase activity in *Escherichia coli* using the gene-protein database

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**Abstract**

The gene-protein database was used to obtain the two-dimensional polyacrylamide gel coordinates of proteins phosphorylated in extracts of *Escherichia coli* including those phosphorylated by eukaryotic-like kinase activities. These suggest that the phosphoproteins correspond to, or co-migrate with, the product of an open reading frame at 1.3 min (Orf80), Enzyme 1 of the phosphoenolpyruvate-dependent phosphotransferase system (PtsI), the tRNA synthetase for histidine (HisS), and proteins involved in the response to carbon starvation and quinone treatment.

**Keywords:** Gene-protein index; *Escherichia coli*; Protein kinase; Calcium

**1. Introduction**

In *Escherichia coli*, the majority of the 130 or so proteins phosphorylated in vivo are phosphorylated on serine, threonine or tyrosine as in eukaryotes, and the majority of these await identification ([1]; but see [2]). The few eukaryotic-like kinases and their substrates identified in this leading model system include elongation factor Tu [3], which in eukaryotes is a substrate of protein kinase C (PKC), and heat shock protein DnaK [4,5], which in vitro has an auto-phosphorylation activity stimulated by calcium [6]. Proteins have also been extracted from *E. coli* that cross-react with a variety of antibodies to eukaryotic PKC and that possess an in vitro kinase activity characteristic of eukaryotic PKC, namely, stimulation of phosphorylation by addition of calcium, phosphatidylserine, and phorbol ester [7]. Recently, ribosomal protein S1, succinyl-CoA synthetase and a number of novel proteins have been identified as phosphoproteins following in vitro phosphorylation assays performed on fractionated extracts of *E. coli* [8]; this assay is based on the ion- and lipid-stimulation characteristic of many eukaryotic kinases and, significantly, homologues of S1 and succinyl-CoA synthetase are also phosphorylated in eukaryotes. To identify other ion- and lipid-stimulated kinases and their substrates, we employed two-dimensional polyacrylamide gel analysis in conjunction with the gene-protein database [9] which cross-references many of the known genes and proteins of *E. coli* K12 and permits ready passage from the 2-D coordinates of a protein to a gene carried by a plasmid in a characterized library [10,11].

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2. Materials and methods

Enteropathogenic *E. coli* strain MAR001 and the *E. coli* K12 strain W3110 were used in the study. Bacteria were grown aerobically at 37°C in Luria broth containing 0.2% (w/v) glucose to an OD₆₀₀ of 0.7 as previously described [7]. 1 l of cells was harvested, washed once with phosphate-buffered saline and once with 20 mM Tris·HCl (pH 7.5) and resuspended in 20 mM Tris·HCl (pH 7.5), 1 mM leupeptin, 1 mM chymostatin, 1 mM elastatinal, 1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N',N',N' -tetraacetic acid (EGTA) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were lysed by sonication and the lysates spun in a Microcentaur bench top centrifuge at 10000 × g to yield ‘+M’ extracts; in some experiments, these extracts were spun in a Beckman TL-100 rotor at 57000 × g for 30 min at 4°C, to remove membrane and yield ‘−M’ extracts. The supernatants were used for the assay as described previously [7]; each assay comprised 20 mM Tris·HCl (pH 7.5), 5 mM MgCl₂, 0.185 mBq [γ-³²P]ATP in 1 μM unlabelled ATP, and 5 μl of sonicate in a final volume of 50 μl. Hence all assays were performed in at least 0.1 mM EGTA. Where appropriate, other final concentrations were CaCl₂, 4 mM, and phosphatidylserine, 40 μg ml⁻¹. Assay mixtures were incubated at 37°C for 15 min and reactions were stopped by addition of 30 μl of 5 × SDS-PAGE sample buffer; samples were then heated to 95°C for 5 min and 40 μl were used for SDS-PAGE.

For 2-D gels, phosphorylation reactions were stopped by addition of 30 μl of isoelectric focusing
sample buffer. Samples were then loaded onto isoelectric focusing rods of the Millipore 'Investigator' system, prepared and analysed as previously described [9]. In parallel, phosphorylation assay samples were analysed which contained 2 μl of [35S]methionine labelled lysate of an E. coli reference strain.

3. Results and discussion

To help identify proteins phosphorylated by eu-karyotic-like kinase activity, the reference pattern of [35S]methionine-labelled polypeptides in the gene-protein database (Fig. 1) was compared with the pattern of γ-32P-labelled proteins obtained in our assay on lysates of the enteropathogenic strain, MAR001, with membrane present (Fig. 2). Table 1 shows the results for the K12 reference strain, W3110, and for the strain, MAR001, in each case with and without membranes (+M and −M extracts, respectively). To aid identification, the 35S- and 32P-labelled materials were also mixed and analysed by 2-D analysis (data not shown).

Eleven phosphorylated proteins were resolved by 2-D gel electrophoresis and their co-ordinates in the gene-protein database are given in Table 1. Plasmids in the Clarke and Carbon library that carry a gene encoding a protein with similar coordinates are also listed in Table 1.

Fig. 2. 2-D analysis of kinase activity in presence of CaCl₂ and phosphatidylserine in sonicates of enteropathogenic E. coli strain MAR001 with membrane.
Protein a, with coordinates 91.5 × 154.5 and designation B82.5, was phosphorylated in all extracts; note that protein a appears more strongly labelled than b in extracts of the MAR001 (Fig. 2) but not in the W3110 strains (data not shown). Phosphorylation of protein a also appeared greater in the +M extract (Fig. 2) of MAR001 than in the −M extract (data not shown). Protein a corresponds to, or co-migrates with, a protein encoded by a gene on plasmid pLC28-25. The insert on this plasmid has been mapped to 1.3 min on the E. coli genome [12]; this region has been sequenced and an open reading frame, orf80, identified that would encode a protein of around 85 kDa with no obvious homology to any known protein [13]. Phosphorylation of Orf80 was stimulated by addition of calcium and inhibited by further addition of the ion chelator EGTA (data not shown).

Protein b, with coordinates 98 × 142 and designation B058.3, was phosphorylated in all extracts. It co-migrates with a known phosphoprotein – Enzyme 1 of the phosphoenolpyruvate-dependent phosphotransferase system, PtsI [14]. In some experiments, addition of calcium appeared to inhibit phosphorylation of this protein (data not shown).

Protein c, with coordinates 72 × 134 and designation E53.7, and protein d, with coordinates 85 × 133.5 and designation C51.00, were phosphorylated in all extracts. Protein d corresponds to, or co-migrates with, a protein encoded by a gene on plasmid pLC28-24 and induced by carbon starvation [15]. Phosphorylation of c and/or d was inhibited by EGTA (data not shown).

Protein e, with coordinates 53 × 129.5 and designation F48.1, was phosphorylated in Mar001 extracts but not in W3110 extracts. It corresponds to, or co-migrates with, HisS the tRNA synthetase for histidine. Phosphorylation was strongly inhibited by addition of EGTA; addition of phosphatidylserine had no effect (data not shown). Two other tRNA synthetases in E. coli have been reported as phosphorylated on serine/threonine residues whilst in the cells of higher eukaryotes, namely Chinese hamster ovary cells, histidyl tRNA synthetase is also phosphorylated on serine [16]. In the lower eukaryote, Saccharomyces cerevisiae, both the endogenous and the E. coli histidyl tRNA synthetase have a remarkable homology with the GCN2 kinase which plays a key role in the response to amino acid starvation [17]. Eukaryotic initiation factor, eiF2-α, is a sub-strate of GCN2-like kinases and it may be significant that eiF2-α has considerable homology to the E. coli ribosomal protein S1 which is phosphorylated by a eukaryotic kinase-like activity [8].

Protein f, with coordinates 56.5 × 125.5 and designation E46.7, which was phosphorylated in all extracts, migrated as a streak in the iso-electric focusing dimension.

Protein g, with coordinates 80 × 121.5 and designation C41.0, and protein h, with coordinates 83 × 121.5, were phosphorylated in all extracts.

Protein i, with coordinates 45.5 × 97 and designation B82.5, was phosphorylated in all extracts; note that protein i appears more strongly labelled than j in extracts of the MAR001 (Fig. 2) but not in the W3110 strains (data not shown). Phosphorylation of protein i also appeared greater in the +M extract (Fig. 2) of MAR001 than in the −M extract (data not shown). Protein i corresponds to, or co-migrates with, a protein encoded by a gene on plasmid pLC28-25. The insert on this plasmid has been mapped to 1.3 min on the E. coli genome [12]; this region has been sequenced and an open reading frame, orf80, identified that would encode a protein of around 85 kDa with no obvious homology to any known protein [13]. Phosphorylation of Orf80 was stimulated by addition of calcium and inhibited by further addition of the ion chelator EGTA (data not shown).

Protein j, with coordinates 54 × 82.5, was phosphorylated in all extracts; note that protein j appears more strongly labelled than k in extracts of the MAR001 (Fig. 2) but not in the W3110 strains (data not shown). Phosphorylation of protein j also appeared greater in the +M extract (Fig. 2) of MAR001 than in the −M extract (data not shown). Protein j corresponds to, or co-migrates with, a protein encoded by a gene on plasmid pLC28-25. The insert on this plasmid has been mapped to 1.3 min on the E. coli genome [12]; this region has been sequenced and an open reading frame, orf80, identified that would encode a protein of around 85 kDa with no obvious homology to any known protein [13]. Phosphorylation of Orf80 was stimulated by addition of calcium and inhibited by further addition of the ion chelator EGTA (data not shown).

Protein k, with coordinates 105.5 × 61.5, was phosphorylated in all extracts; note that protein k appears more strongly labelled than l in extracts of the MAR001 (Fig. 2) but not in the W3110 strains (data not shown). Phosphorylation of protein k also appeared greater in the +M extract (Fig. 2) of MAR001 than in the −M extract (data not shown). Protein k corresponds to, or co-migrates with, a protein encoded by a gene on plasmid pLC28-25. The insert on this plasmid has been mapped to 1.3 min on the E. coli genome [12]; this region has been sequenced and an open reading frame, orf80, identified that would encode a protein of around 85 kDa with no obvious homology to any known protein [13]. Phosphorylation of Orf80 was stimulated by addition of calcium and inhibited by further addition of the ion chelator EGTA (data not shown).

Table 1
Summary of phosphoprotein characteristics as determined by coordinates on the gene-protein database

<table>
<thead>
<tr>
<th>Coordinates</th>
<th>W3110 + M</th>
<th>W3110 − M</th>
<th>MAR + M</th>
<th>MAR − M</th>
<th>pI</th>
<th>M, A−N</th>
<th>C.&amp; C. ref.</th>
</tr>
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<tbody>
<tr>
<td>a 91.5 × 154.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5.11</td>
<td>82.5</td>
<td>B82.5</td>
</tr>
<tr>
<td>b 98 × 142</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>4.97</td>
<td>63.5</td>
<td>B058.3</td>
</tr>
<tr>
<td>c 72 × 134</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5.52</td>
<td>53.6</td>
<td>E53.7</td>
</tr>
<tr>
<td>d 85 × 133.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5.25</td>
<td>51</td>
<td>E53.7</td>
</tr>
<tr>
<td>e 53 × 129.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5.91</td>
<td>48</td>
<td>E48.1</td>
</tr>
<tr>
<td>f 56.5 × 125.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5.84</td>
<td>4.67</td>
<td>E46.7</td>
</tr>
<tr>
<td>g 80 × 121.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5.35</td>
<td>44.4</td>
<td>C41.0</td>
</tr>
<tr>
<td>h 83 × 121.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5.29</td>
<td>44.4</td>
<td>C41.0</td>
</tr>
<tr>
<td>i 45.5 × 97</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>6.07</td>
<td>32</td>
<td>G32.0</td>
</tr>
<tr>
<td>j 54 × 82.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5.89</td>
<td>25.5</td>
<td>C41.0</td>
</tr>
<tr>
<td>k 105.5 × 61.5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>4.83</td>
<td>19</td>
<td>C41.0</td>
</tr>
</tbody>
</table>

+, phosphoprotein present; M, membrane; A−N, alphanumeric designation in the gene-protein database; C. and C. ref., reference of plasmid in Clarke and Carbon library encoding polypeptide with corresponding coordinates in gene-protein database.
tion G32.00 was phosphorylated in all extracts. G32.0 corresponds to, or co-migrates with, a quinone induced protein encoded by a gene carried on pLC28-24, to which C51.00 has also been assigned [15]. On the reference gel (Fig. 1), the closest radioactive spots to G32.00 correspond to isoforms of the autophosphorylated, Ras-like protein of E. coli, Era [18].

Proteins j and k, with coordinates 54 × 82.5 and 105.5 × 61.5, respectively, were phosphorylated in +M and −M extracts of W3110 and only +M (Fig. 2) extracts of MAR001. Phosphorylation of k was slightly stimulated by the addition of phosphatidylserine (data not shown).

There are several important caveats. Firstly, EGTA chelates a variety of ions such as manganese, zinc and iron better than calcium and the reversal of the effects of EGTA by addition of calcium may be due to release of one of these other ions [19]; in a separate series of experiments, we have discovered stimulation of different kinase activities by calcium, manganese and zinc [8]. Secondly, phosphorylation in vitro may not always reflect phosphorylation in vivo; nevertheless, two of the proteins identified here and seven of the proteins we have identified elsewhere are known to be phosphorylated in vivo [8]. Thirdly, using the gene-protein database to identify phosphoproteins requires caution since modifications in the isoelectric point of proteins due to phosphorylation may alter 2-D coordinates especially in the lower M, range. This is only a problem if the protein is not phosphorylated in the in vivo conditions used to establish the gene-protein database. Moreover, in our experience, phosphorylation often has no effect on 2-D coordinates. For example, phosphorylated S1 co-migrates with unphosphorylated S1 [8], a known phosphoprotein, PtsI, co-migrates with protein b and isoforms of another known phosphoprotein, Era, are represented in the gene-protein database as the closest spots to protein i. Fourthly, stimulation of kinase activity may vary because a large number of factors determine protein phosphorylation and its pattern therefore shows a chaotic dependence. Further study of proteins a to k should lead to clarification of the relationship of ion and lipid addition to phosphorylation of the purified proteins, confirmation of phosphorylation in vivo and the physiological relevance of such phosphorylation.

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References


