Protein tyrosine kinases in bacterial pathogens are associated with virulence and production of exopolysaccharide

Ofir Ilan, Yafa Bloch, Gad Frankel¹,
Henriette Ullrich², Klaus Geider² and
Ilan Rosenshine³

Department of Molecular Genetics and Biotechnology, The Hebrew University Faculty of Medicine, PO Box 12272, Jerusalem 91120, Israel, ¹Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AZ, UK and ²Max-Planck-Institut Fur Zellbiologie, Rosenhof, D-68526 Ladenburg, Germany

³Corresponding author
e-mail: ilanro@cc.huji.ac.il

In eukaryotes, tyrosine protein phosphorylation has been studied extensively, while in bacteria, it is considered rare and is poorly defined. We demonstrate that *Escherichia coli* possesses a gene, *etk*, encoding an inner membrane protein that catalyses tyrosine autophosphorylation and phosphorylation of a synthetic co-polymer poly(Glu:Tyr). This protein tyrosine kinase (PTK) was termed Ep85 or Etk. All the *E.coli* strains examined possessed *etk*; however, only a subset of pathogenic strains expressed it. Etk is homologous to several bacterial proteins including the Ptk protein of *Acinetobacter johnsonii*, which is the only other known prokaryotic PTK. Other Etk homologues are AmsA of the plant pathogen *Erwinia amylovora* and Orf6 of the human pathogen *Klebsiella pneumoniae*. These proteins are involved in the production of exopolysaccharide (EPS) required for virulence. We demonstrated that like Etk, AmsA and probably also Orf6 are PTKs. Taken together, these findings suggest that tyrosine protein phosphorylation in prokaryotes is more common than was appreciated previously, and that Etk and its homologues define a distinct protein family of prokaryotic membrane-associated PTKs involved in EPS production and virulence. These prokaryotic PTKs may serve as a new target for the development of new antibiotics.

Keywords: AmsA/*Erwinia amylovora*/Etk/
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Introduction

Tyrosine protein phosphorylation and its biological significance have been characterized extensively in eukaryotes (a few recent reviews include Hubbard *et al.*, 1998; Schlaepfer and Hunter, 1998; Tzahar and Yarden, 1998; Williams *et al.*, 1998). In contrast, only a few reports describe tyrosine protein phosphorylation in prokaryotes (Atkinson *et al.*, 1992; Kelly-Wintenberg *et al.*, 1993; South *et al.*, 1994; Frasch and Dworkin, 1996), and one report describes a prokaryotic protein tyrosine kinase (PTK): the Ptk protein of *Acinetobacter johnsonii* (Grangeasse *et al.*, 1997). Except for Ptk, none of the

bacterial tyrosine-phosphorylated proteins or the corresponding kinases were identified. Moreover, the biological significance of these tyrosine protein phosphorylation events remained elusive. Thus, tyrosine protein phosphorylation in prokaryotes is regarded as rare and is poorly defined (Zhang, 1996; Cozzzone, 1998).

Exopolysaccharides (EPSs) are important virulence factors of many animal and plant pathogens. The role of the EPSs in forming a capsule that protects the pathogen from phagocytosis is well documented (Ofek *et al.*, 1993). However, EPSs may contribute to virulence in other ways. In *Erwinia amylovora* and *Ralstonia solanacearum*, the EPS appears to be required for initial attachment of these pathogens to the host plant tissue (Cook and Sequeira, 1991; Bugert and Geider, 1995), a step which may be needed for efficient delivery of virulence factors via the type III secretion systems. In *Pseudomonas aeruginosa*, the loose EPS aggravates the lung infections in cystic fibrosis patients. In the plant symbiont *Rhizobium meliloti*, the succinoglycan EPSs play a more specific role in interaction with the host plant. The succinoglycan is sloughed off into the surroundings and mediates signalling to the host cell which is essential for the formation of fully matured nodules (Leigh and Walker, 1994).

We reported previously that enteropathogenic *Escherichia coli* (EPEC) possesses a tyrosine-phosphorylated protein, Ep85 (Rosenhine *et al.*, 1992). In this communication, we describe the purification of Ep85 which we renamed Etk, and the characterization of Etk and its coding gene. We show that Etk is a membrane-associated PTK that is expressed specifically by pathogenic strains of *E.coli*. In addition, we demonstrate that Etk is closely related to the AmsA protein of the plant pathogen *E.amylovora* and the Orf6 protein of human pathogen *Klebsiella pneumoniae*. Both Orf6 and AmsA are involved in virulence and production of EPS.

Results

Identification of tyrosine-phosphorylated protein in EPEC and its coding gene

While studying aspects of the virulence of EPEC, we noticed that this pathogen possesses a protein, Ep85, that cross-reacted with several monoclonal anti-phosphotyrosine antibodies, including PY20, 4G10 and PT66 (Rosenhine *et al.*, 1992; data not shown). We purified Ep85 and determined the amino acid sequence of its N-terminus, which is MTTKNMNTPPGSTQENE. This sequence matched perfectly with the N-terminal sequence of an *E.coli* putative protein YccC encoded by an open reading frame (ORF) at the *appA* 3' region of the *E.coli* K12 MG1655 genome (Blattner *et al.*, 1997). This putative protein is 81.2 kDa in size, similar to the apparent molecular size of Ep85, as determined by SDS-PAGE (Rosenhine

A

B

EGFR_HUMAN 1 FKKIKVLGSGAGTGVYKGLMPEGEKVKIPVAKEKREATSPKANKEILDEAYVMSVDNPHVCRLLGICLTSTVQLITQLMPFGCLLDYVREHKDNIGSYQLL 104
 Etk 597 LNKVIQHFQKGGFVDITRGQVFPNPSELNKRDRMRQLLEWANDHYLDVDTFPMPLAVSDAAVGRSVGTSLLVAFPGINTAKEVSLSMQRLEQAGVNTKGAIL 700

Fig. 1. Amino acid sequence of Etk and comparison between some Etk homologues. (A) Alignment of the amino acid sequence of Etk and several Etk homologues including Wzc of *E.coli*, Orf6 of *K.pneumoniae*, AmsA of *E.amylovora* and EpsB of *R.solanacearum*. Identity is indicated by (*), higher and lower similarities are indicated by (:) and (.), respectively. A nucleotide-binding motif is highlighted and three potential transmembrane helices are underlined and highlighted. (B) The homology between a segment from the catalytic domain of EGFR and an C-terminal segment of Etk.

et al., 1992). We amplified a DNA fragment from EPEC E2348/69 that encodes Ep85 and cloned it under the control of the *tac* promoter, adding in the process a His tag at its N-terminus. The nucleotide sequence of the Ep85-encoding gene from EPEC (DDBJ/EMBL/GenBank accession No. AJ238695), was found to be 97.6% identical to the nucleotide sequence of the corresponding ORF in *E.coli* K12 gene. It encodes a protein 80.5 kDa in size that differs by three residues from the corresponding ORF in *E.coli* K12 (L92, G169 and G216 in the EPEC protein instead of Q92, E169 and E216, respectively, in the K12 protein). Both proteins contain the nucleotide-binding motif AXXXXGKT (Figure 1A).

Ep85 catalyses tyrosine autophosphorylation

As found by many others, we also could not detect tyrosine-phosphorylated proteins in *E.coli* K12, using anti-phosphotyrosine antibodies (data not shown; Figure 5). In contrast, recombinant Ep85 expressed in *E.coli* K12 strains was detected with anti-Ep85 antibody that we generated, and with anti-phosphotyrosine antibody (Figure 2A). This indicates that *in vivo*, Ep85 becomes tyrosine phosphorylated in *E.coli* K12. To demonstrate that Ep85 catalyses tyrosine autophosphorylation, the recombinant Ep85 was expressed in *E.coli* K12, affinity purified and used in a protein kinase (PK) assay with [γ - 32 P]ATP as a phosphate donor. The purified Ep85 exhibits a rapid autophosphorylation activity, suggesting that it is an ATP-dependent PK (Figure 2B). Accordingly, the purified phosphorylated Ep85 was dephosphorylated rapidly by the specific tyrosine protein phosphatase YopH (Figure 2C). In addition, phosphoamino acid analysis indicated that the only phos-

phorylated residues in Ep85 were tyrosines (Figure 2D). Thus, we renamed Ep85 as Etk (for *E.coli* protein tyrosine kinase), and the gene encoding it was renamed *etk*.

Etk catalyses tyrosine phosphorylation of exogenic substrate

The pattern of the proteins that were recognized by anti-Ep85 and anti-phosphotyrosine antibody in a crude extract of *E.coli* K12 expressing Etk was not identical. Some of these differentially recognized proteins were smaller than Etk and may represent degradation products of Etk. However, one of these proteins was larger than Etk and may represent an authentic phosphorylation substrate (Figure 2A). We further investigated the ability of Etk to phosphorylate *in vitro* the exogenic substrate poly(Glu:Tyr) co-polymer. Affinity-purified Etk was incubated with [γ - 32 P]ATP as a phosphate donor and poly(Glu:Tyr) as phosphate acceptor, and phosphorylation of poly(Glu:Tyr) was monitored. Rapid tyrosine phosphorylation of poly(Glu:Tyr) was detected (Figure 3). Taken together, these results indicate the Etk carries out tyrosine phosphorylation of exogenic substrates.

Etk is an inner membrane protein

We next used cell fractionation and immunoblot analysis to determine in which cellular compartment Etk resides. These experiments indicate that most of the Etk was associated with the inner membrane and only a small fraction of it was soluble (Figure 4). In agreement with its membrane localization, the prediction carried out using the MOMENT program, based on the average hydrophobicity of a sliding window of size 21, identified three

Fig. 2. Ep85/Etk catalyses tyrosine autophosphorylation. (A) *Escherichia coli* K12 MC1061, that does not normally express Etk (Figure 5), was transformed with pOI194. The culture was grown to OD = 0.6 and IPTG was added to a final concentration of 0.1 mM. The culture was sampled at 0, 5, 10, 20 and 30 min after IPTG was added, and bacterial proteins were extracted and used for immunoblot analysis with anti-Etk and anti-phosphotyrosine antibodies. Tyrosine-phosphorylated Etk (arrowhead) was detected even before the addition of IPTG, representing the normal basal level of *Ptac* activity. IPTG induction increased expression of phosphorylated Etk, and phosphorylation of an additional protein, larger than Etk, that was not detected with the anti-Etk antibody (arrow). (B) Purified Etk was used for autophosphorylation assay with [γ - 32 P]ATP as a phosphate donor. The reaction was stopped after 0, 15, 30, 45, 60, 75, 90 and 120 s and aliquots were analysed by SDS-PAGE and autoradiography (panel 1), and by immunoblot analysis with anti-Etk to verify an equal amount of Etk in the different aliquots (panel 2). Rapid autophosphorylation of Etk was detected. (C) Purified tyrosine-phosphorylated Etk was treated with YopH, a specific tyrosine protein phosphatase. The reaction was stopped 30, 60 and 90 s after adding YopH and aliquots were subjected to immunoblot analysis with anti-Etk to verify an equal amount of Etk and with anti-phosphotyrosine antibody to determine the tyrosine phosphorylation levels. Rapid tyrosine dephosphorylation of Etk was detected. (D) Purified Etk was labelled with [γ - 32 P]ATP, hydrolysed and phosphoamino acids were analysed by cellulose TLC. The position of phosphoamino acid markers (P-Ser, P-Thr and P-Tyr) as detected by ninhydrin staining of the TLC plate is shown in lane 1. The corresponding autoradiogram of the TLC plate is shown in lane 2. All the labelling was associated with phosphotyrosine but not with phosphothreonine or phosphoserine.

potential transmembrane helices in Etk: from F33 to T53, A425 to A445 and Y641 to G661 (Figure 1A). The membrane localization of Etk may suggest that it is involved in signalling or in transport processes.

Differential expression and phosphorylation of Etk during EPEC growth

We determined the expression pattern of Etk and its tyrosine phosphorylation levels during EPEC growth.

Fig. 3. Etk phosphorylates poly(Glu:Tyr). (A) Poly(Glu:Tyr) at concentrations of 0.008–2 mg/ml was used as an exogenous substrate for Etk. The reaction was carried out for 30 min at 24°C. (B) Poly(Glu:Tyr) at a concentration of 1 mg/ml was used as the Etk substrate. The reaction was carried out for the indicated time at 30°C; 1 pmol of ATP corresponds to ~400 c.p.m.

Fig. 4. Localization of Etk to the inner membrane. An EPEC culture, grown to the mid-logarithmic growth phase, was fractionated into three fractions containing (i) cytoplasmic and periplasmic soluble proteins (S-fraction), (ii) outer membrane proteins (O-fraction) and (iii) inner membrane proteins (I-fraction). The amount of proteins in each fraction was normalized, and proteins were used for immunoblot analysis with different antibodies as indicated beneath each panel. Anti-phosphotyrosine and anti-Etk antibodies detected most of the Etk in the I-fraction and only a small amount of it in the S-fraction. Anti-DnaK, anti-FtsH and anti-intimin antibodies were used as controls to determine the quality of the fractionation. Anti-DnaK and anti-FtsH antibodies reacted as expected with soluble or inner membrane proteins of the same size as DnaK and FtsH. Intimin is an outer membrane protein and is subject to N-terminal processing upon secretion. As expected, the anti-intimin antibody reacted with processed intimin in the O-fraction and with the pre-processed intimin and a small amount of processed intimin in the I-fraction. Molecular weight markers are shown on the left.

While the level of Etk declines upon entering the stationary phase, the levels of the phosphorylated form of the protein remained constant (Figure 5). This may suggest that only an unphosphorylated subpopulation of Etk was targeted for degradation. In addition, at different stages of EPEC

Fig. 5. Expression of Etk during EPEC growth. EPEC growth in LB broth, 37°C, with shaking was monitored by measuring the OD₆₀₀ of the culture. This culture was sampled at 30 min intervals and proteins were extracted. Equal protein concentrations were used for immunoblot analysis with anti-phosphotyrosine and anti-Etk antibodies. The time values on the x-axis of the growth curve correspond also to the immunoblot beneath the axis. Molecular weight markers are shown on the left-hand side. Insert: demonstration that the anti-Etk recognized Etk epitopes other than phosphotyrosine. This control was needed since anti-Etk antibodies were generated by injecting rabbits with phosphorylated Etk. Proteins were extracted from mid-logarithmic EPEC cultures, and were used for immunoblot analysis with anti-phosphotyrosine antibody (PT66, Sigma) (lane 1); PT66 supplemented with 10 mM phenyl phosphate (lane 2); non-specific rabbit antiserum (pre-bleed of anti-Etk) (lane 3); anti-Etk antibody (lane 4); and anti-Etk antibody supplemented with 50 mM phenyl phosphate (lane 5). Molecular weight markers are shown on the left-hand side. The phenyl phosphate, which is identical to the side chain of the tyrosine-phosphorylated residue, fully out-competed the labelling with PT66 but not with anti-Etk antibody. This indicates that anti-Etk reacted with Etk epitopes other than the tyrosine-phosphorylated residues.

growth, changes in the mobility of Etk on SDS-PAGE were observed consistently, and the Etk bands exhibit an irregular M-shape (Figure 5). This suggests that Etk may be modified further at late growth phases. This mobility shift and the irregular band shape were never observed when recombinant Etk was expressed in *E. coli* K12 (Figure 2A and B; data not shown). These results indicate that Etk expression and its post-translational modifications are dynamic processes during EPEC growth.

Etk is expressed by a subset of pathogenic strains of *E. coli*

We examined the presence of *etk* and its expression in six clinical isolates of EPEC and several *E. coli* K12 strains (Figure 6A). In all the strains, the specific *etk* primers amplified one fragment identical in size to *etk*. This PCR analysis indicated that all the EPEC and *E. coli* K12 strains possess the *etk* gene. In contrast, only the EPEC strains express the Etk protein (Figure 6A). We performed the same analysis with several diarrhoeagenic virotypes of *E. coli*, including enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC) and enteroinvasive *E. coli* (EIEC). Again, the *etk* gene was detected in all of the *E. coli* strains examined

Fig. 6. Expression of Etk by different *E. coli* strains. Proteins were extracted from cultures of different *E. coli* strains grown to OD₆₀₀ = 1.0. Etk expression and its phosphorylation state were analysed by immunoblotting with anti-Etk and anti-phosphotyrosine antibodies. In addition, DNA was extracted from the different strains and the existence of the *etk* gene was examined by PCR with specific *etk* primers. (A) Six clinical isolates of EPEC, including E815/71, O142:H6 (lane 1), E2348/69, O127:H6 (lane 2), 2430/78, O111ab:NM (lane 3), 1092-80, O127:NM (lane 4), 2087-77, O55:H6 (lane 5) 0659-79, O119:H6 (lane 6), and three non-pathogenic *E. coli* laboratory strains including MC1061 (lane 7), K91 (lane 8) and HB101 (lane 9) were tested. The specific *etk* primers amplified *etk* in all the strains but it was expressed only in the EPEC isolates. (B) Different *E. coli* virotypes were compared including EIEC E5273/0, O28ac:H⁻ (lane 1), EAEC 17-2, O3:H2 (lane 2), ETEC H10407, O78:H11 (lane 3) and EPEC E2348/69 (lane 4). As before, the PCR analysis indicates that all strains possess *etk* but only EPEC and ETEC express it.

but was expressed only by EPEC, ETEC (Figure 6B) and EHEC (data not shown). The molecular basis for the differential expression of *etk* between different *E. coli* strains has yet to be determined. Expression of Etk by only a subset of pathogenic strains of *E. coli* suggests that Etk may play a part in virulence mechanisms.

Etk is a member of a protein family

Etk is homologous to several bacterial proteins that are involved in the production of EPSs. The Etk homologues include Wzc, an Etk-like protein in *E. coli* K12 that is involved in the production of a colanic acid capsule (Stevenson *et al.*, 1996); Orf6 of *K. pneumoniae* which is required for the formation of a K2 capsule (Arakawa *et al.*, 1995); AmsA of *E. amylovora* which is required for amylovoran production (Bugert and Geider, 1995); EpsB of *R. solanacearum*, which is needed for the production of EPS-I (Cook and Sequeira, 1991), and ExoP of *R. meliloti*, which is needed for production of succinoglycan (Becker *et al.*, 1993) (Figure 1; data not shown). The K2 capsule, amylovoran, EPS-I and succinoglycan are required for virulence or interaction of the corresponding pathogens with the respective animal or plant host. Wzc, Orf6, AmsA and EpsB exhibit >50% identity and 70% similarity to Etk, while ExoP is somewhat less similar. Etk is also ~36% identical and 40% similar to Ptk, the only known prokaryotic PTK of *A. johnsonii* (Grangeasse *et al.*, 1997).

Fig. 7. Expression of Etk homologues by *K.pneumoniae* and *E.amylovora*. (A) Proteins were extracted from EPEC 2348/69 (lane 1) or *K.pneumoniae* K2 KPA1 (lane 2), both grown on LB, and from *E.amylovora* Ea7/74 (lane 4) or the isogenic *amsA* mutant Ea7/74-A56 (lane 3), both grown on LB supplemented with 0.2% sorbitol. The extracted proteins were used for immunoblot analysis with anti-Etk supplemented with 10 mM phenyl phosphate and with anti-phosphotyrosine antibody. The anti-Etk antibody reacted with proteins of similar size to Orf6 and AmsA in the *K.pneumoniae* and *E.amylovora* extracts (arrows). These proteins also reacted with the anti-phosphotyrosine antibody (arrows). In contrast, neither of these antibodies reacted with corresponding protein in the *E.amylovora amsA* mutant. This indicates that like Etk, Orf6 and AmsA are tyrosine phosphorylated and probably are PTKs. (B) A recombinant AmsA was expressed in *E.coli* K12 XL1 Blue/pfdC4Z-*amsA* (lane 1), and proteins were extracted and analysed with anti-Etk and anti-phosphotyrosine antibodies. As a negative control we used XL1 Blue (lane 2) and as positive control we used XL1 Blue transformed with pOI194 that express Etk (lane 3). AmsA reacted specifically with anti-Etk and anti-phosphotyrosine.

***E.amylovora* and *K.pneumoniae* express tyrosine-phosphorylated proteins that cross-react with anti-Etk antibody**

We predicted that like Etk and Ptk, other Etk homologues are also PTKs. To test this prediction, we analysed extracts of *E.amylovora* and *K.pneumoniae* by immunoblot analysis with anti-phosphotyrosine and anti-Etk antibodies. In agreement with our prediction, *E.amylovora* Ea7/74 contained a protein similar in size to AmsA that cross-reacts with anti-Etk and with anti-phosphotyrosine antibody (Figure 7A). Moreover, this cross-reaction was not detected in the isogenic *E.amylovora amsA* mutant strain, Ea7/74-A56 (Figure 7A). The same results were obtained with a different *E.amylovora* isolate, strain 1/79, and its isogenic *amsA* mutant strain 1/79-D49 (data not shown). *Klebsiella pneumoniae* K2 KPA1 (Ofek *et al.*, 1993) also contained a protein, presumably Orf6, that cross-reacted with anti-phosphotyrosine and anti-Etk antibodies (Figure 7A). These results support the suggestion that AmsA and Orf6 are PTKs. Interestingly, like Etk, both AmsA and Orf6 exhibit a higher molecular weight than expected and an M-like band shape in SDS-PAGE (Figure 7). These characteristics were never observed in recombinant AmsA expressed in *E.coli* K12 (Figure 7B).

Table I. Complementation of an *E.amylovora amsA* mutant

	Ea7/74	Ea7/74-A56	Ea7/74-A56 (pfdC4Z'- <i>amsA</i> 1)
EPS on MM1gal-agar, colony morphology ^a	muc	nmu	muc
EPS in MM1gal ⁻ medium (μg/ml) ^b	160	15	155
Ooze on pear slices ^c	+	-	+
Sensitivity to Ea1h ^d	+	-	+

^aMM1, the minimal medium has been described in Bugert and Geider (1995); gal, 1% galactose as carbon source; muc, mucoid; nmu, non-mucoid.

^bEPS production was measured by the CPC turbidity assay (Bugert and Geider, 1995).

^cVirulence was assayed by production of ooze on pear slices as described previously (Bernhard *et al.*, 1993).

^dAmylovoran-specific *E.amylovora* phage.

AmsA of *E.amylovora* is a PTK

We cloned the *amsA* gene under the control of the *lac* promoter in the plasmid pfdC4Z-*amsA*. This plasmid was introduced into *E.amylovora amsA* mutant Ea7/74-A56 and it restored the wild-type phenotype including formation of mucoid colonies, production of amylovoran EPS, sensitivity to the EPS-specific phage Ea1h and generation of ooze on slices of infected immature pears (Table I). We introduced pfdC4Z-*amsA* into *E.coli* K12 XL1-Blue, induced AmsA expression with isopropyl-β-D-thiogalactopyranoside (IPTG) and extracted the bacterial proteins. The extracted proteins were analysed by immunoblot analysis with anti-phosphotyrosine and anti-Etk antibodies. The recombinant AmsA reacted specifically with these antibodies (Figure 7B), indicating that the recombinant AmsA catalyses tyrosine autophosphorylation *in vivo*.

Discussion

Etk, Ptk, AmsA, Orf6 and related proteins appear to form a family of prokaryotic PTKs. AmsA and Orf6 are required for EPS production and for virulence. *Erwinia amylovora amsA* mutants show reduced EPS production, and the virulence of these mutants on immature pear fruits is strongly attenuated (Bugert and Geider, 1995). It was suggested that EPS is important in mediating the initial attachment of *E.amylovora* to the host plant tissues. In *K.pneumoniae* K2, the EPS forms a large K2 capsule while *orf6* mutants do not form this capsule (Arakawa *et al.*, 1995). The K2 capsule prevents phagocytosis by macrophages, and non-capsulated mutants are much more sensitive to phagocytosis than the wild-type strain (Ofek *et al.*, 1993). Moreover, the K2 capsule type frequently is associated with *K.pneumoniae* pathogenicity in humans. In the mouse animal model, the LD₅₀ of non-capsulated mutants is reduced by more than three orders of magnitude in comparison with that of the wild-type strain (Ofek *et al.*, 1993; Arakawa *et al.*, 1995). In *R.meliloti*, EPS plays a more specific role in host-symbiont interaction. In this case, the succinoglycan EPS is sloughed off into the surroundings and plays a role in specific signalling to the host plant cells (Leigh and Walker, 1994). The correlation between Etk expression and specific *E.coli* virotypes including EPEC, EHEC and ETEC suggests some role for Etk in virulence. We currently are using an *etk* knock-

out mutant of EPEC to test this hypothesis. Preliminary results indicate that Etk is not involved in formation of attaching and effacing lesions to tissue culture cell lines.

We demonstrated that Etk can catalyse tyrosine phosphorylation of exogenic substrates. However, we still need to identify a genuine physiological exogenic protein substrate in order to define Etk and AmsA as authentic eukaryotic-like PTKs. The recently described *E.coli* protein BipA/TypA (Farris *et al.*, 1998; Freestone *et al.*, 1998) may be a specific Etk substrate. This suggestion is supported by the finding that recombinant BipA is tyrosine phosphorylated when expressed in EPEC but not in K12. In addition, tyrosine phosphorylation of BipA is catalysed *in vitro* by EPEC extracts but not by *E.coli* K12 extracts (Freestone *et al.*, 1998).

Extensive comparison of the catalytic domains of PKs (mostly of eukaryotic origin) and structural data have led to the definition of 11 sub-domains (I–XI) that contain conserved elements (Hanks and Hunter, 1995). Grangeasse *et al.* (1997) looked for these conserved features in Ptk of *A.johnsonii* and identified several PK-like regions in Ptk. However, these regions are only partially conserved in Etk and AmsA. For example, no homology could be found between Etk and the 'invariant' I516 and L530 in the putative sub-domain VI of Ptk. On the other hand, the conserved nucleotide-binding motif of Etk and AmsA AXXXGKT (Figure 1) is only partially conserved in Ptk (Figure 1). We detected 20% identity and 40% similarity between a C-terminal segment of Etk and a region that lies in sub-domains I and II (ATP-binding site) of the epidermal growth factor receptor (EGFR) (Figure 1B). However, Etk lacks the third glycine in the GXGXXG motif and the conserved lysine in the VAXK sequence, suggesting that it is unlikely that this is a functionally meaningful homology. In conclusion, we could not demonstrate any clear homology between Etk or AmsA and eukaryotic PTKs.

The lack of distinct homology with eukaryotic PTKs may indicate that Etk and its prokaryotic homologues utilize a catalytic mechanism different from that of the eukaryotic PTKs. We speculate that the catalytic domain of Etk is in a C-terminal domain that contains the nucleotide-binding motif and that it lies within a cytoplasmic loop between two membrane domains. However, structure–function analysis is needed to test this prediction and for analysis of the catalytic mechanism of Etk. Interestingly, about half of the last 15 C-terminal residues of Etk and all Etk homologues are conserved tyrosines (Figure 1). The significance of this domain and whether it becomes phosphorylated are not yet known.

In *E.coli* K12, the ORF immediately 3' to *etk* encodes a putative protein that is very similar to eukaryotic protein tyrosine phosphatase (PTP) (Blattner *et al.*, 1997). It consists of 152 residues and contains the essential PTP active site residues VCXXXCR (amino acids 16–23) as well as the conserved motif DPY (amino acids 123–125) (Su *et al.*, 1994). Similar putative PTP genes are located adjacent to *amsA*, *orf6*, *epsB*, *exoP*, *ptk* and *wzc* (Cook and Sequeira, 1991; Becker *et al.*, 1993; Arakawa *et al.*, 1995; Bugert and Geider, 1995; Stevenson *et al.*, 1996). Moreover, in the case of *A.johnsonii*, this PTP was demonstrated to dephosphorylate Ptk *in vitro* (Grangeasse *et al.*, 1998). These putative PTP sequences are encoded

by *orf5* and *amsI* in *K.pneumoniae* and *E.amylovora*, respectively (Arakawa *et al.*, 1995; Bugert and Geider, 1995). Both *orf5* and *amsI* are involved in EPS production (Arakawa *et al.*, 1995; Bugert and Geider, 1997). Thus it appears that EPS production is dependent on the activity of PTK and PTP, that form a molecular switch regulated by phosphorylation and dephosphorylation. It remains to be seen how this putative switch governs EPS production.

A third ORF that frequently appears to be associated with all of these prokaryotic PTKs and to be required for EPS production encodes a putative outer membrane lipoprotein (Cook and Sequeira, 1991; Arakawa *et al.*, 1995; Bugert and Geider, 1995; Stevenson *et al.*, 1996; our unpublished observation). Mutants in genes of *R.meliloti* that encode the putative PTK, PTP and the outer membrane lipoprotein (*exoP*, *exoT* and *exoQ*, respectively) still produce the EPS repeating units but do not produce EPS (Reuber and Walker, 1993). Thus, it appears that in *R.meliloti* these proteins are involved in regulating or orchestrating the delivery of the EPS repeating units from the cytoplasm to the extracellular cell surface. Alternatively, these proteins are needed for the polymerization of the secreted repeating units.

In conclusion, the results described here indicate that protein tyrosine phosphorylation in prokaryotes appears to be more common than was appreciated previously. This protein tyrosine phosphorylation is catalysed by a distinct protein family of prokaryotic membrane-associated PTKs that are involved in EPS production, which is essential for the virulence of many pathogens. This family includes Etk, Ptk, AmsA, Orf6 and probably many other related proteins. Many questions are still open: why is *etk* not expressed in all *E.coli* strains? Does Etk play a role in EPEC, EHEC and ETEC virulence? Is Etk involved in signal transduction? What is the catalytic mechanism of Etk and its homologues? How do these PTKs promote EPS production? Resolving these challenging questions may shed new light on some basic aspects of protein tyrosine phosphorylation and its relationship to bacterial physiology and virulence. Moreover, since the EPS is an essential virulence factor of many pathogens, Etk and its homologues represent a new target for the development of specific inhibitors of these prokaryotic PTKs. This putative drug(s) may be used to treat bacterial infections by blocking EPS production.

Materials and methods

Bacterial strains

All pathogenic *E.coli* strains including EPEC, ETEC, EHEC, EAEC and EIEC strains were obtained from M.Donnenberg (The University of Maryland). The *E.coli* K12 strains that were used are common laboratory strains available from different suppliers. The *K.pneumoniae* K2 strain KPA1 (Ofek *et al.*, 1993) was obtained from I.Ofek (Tel-Aviv University). All *E.coli* and *K.pneumoniae* strains were grown in LB agar or LB broth at 37°C. The appropriate antibiotics were added when needed. The *E.amylovora* strains that were used and their growth conditions have been described previously (Bugert and Geider, 1995).

Immunoblot analysis

Samples were subjected to SDS–PAGE and transferred to nitrocellulose membrane (AB-S 83 Schleicher & Schuell Inc.) using a NovaBlot electrophoretic transfer unit (LKB) according to the manufacturer's recommendations (Rosenshine *et al.*, 1992). Monoclonal anti-phosphotyrosine (PT66 Sigma) and polyclonal rabbit anti-Etk, anti-DnaK, anti-FtsH and anti-intimin antibodies were diluted in TBS (150 mM NaCl,

20 mM Tris-HCl pH 7.5) containing 1% bovine serum albumin (BSA; Sigma). Binding of secondary anti-mouse IgG or anti-rabbit IgG (Sigma) alkaline phosphatase-conjugated antibody was detected using the NBT/BCIP (Promega).

Purification and identification of Etk

Etk was purified from an extract of EPEC culture at the mid-logarithmic growth phase by affinity chromatography with agarose-conjugated anti-phosphotyrosine antibody (PT66, Sigma). The eluted protein was resolved by 8% SDS-PAGE, transferred to a PVDF membrane and subjected to N-terminal sequencing using standard procedures.

Recombinant DNA techniques

To clone *etk*, the Ep85-encoding gene, two primers, 5'-ATAAGCTTGCACCTTTTCAGTTTACTCTTTCTCG and 5'-ATGGATCCTATGATACGCCACCAGGCAG, were designed based on a sequence of the *yccC/etk* gene of *E. coli* K12 MG1655 (Blattner *et al.*, 1997). The primers were used to amplify a DNA fragment encoding Ep85 using standard PCR protocols and EPEC E2348/69 chromosomal DNA as a template. The amplified product was digested with *Bam*HI and *Hind*III and ligated into the corresponding sites in pQE31 (Quigene). This generated a plasmid pEP19 expressing, via *Ptac*, a short His tag fused to the N-terminus of full-length Ep85. Overexpression of Ep85 was toxic to the expressing bacteria. To avoid this toxicity and to enable better regulation of the expression of Ep85, a *Sal*I fragment containing the *lacI^q* gene was cloned into the *Xho*I site of pEP19 to generate pOI194. To clone *amsA*, a DNA fragment encoding genomic *amsA* of *E. amylovora* was PCR amplified with the primers 5'-CGCTGCCAGAAATGGG and 5'-GCCATTCATCGTCGGCG, cloned into pGEM-T (Promega), digested with *Sph*I and *Sal*I, and subcloned into plasmid pfdC4Z' under control of the *lac* promoter to give pfdC4Z-*amsA*.

Purification of recombinant proteins

To prepare Ep85/Etk, *E. coli* XL1 Blue containing pOI194 was grown in LB to the mid-logarithmic growth phase and expression of Ep85 was induced by adding IPTG to a final concentration of 0.1 mM. After 2 h expression, Ep85 was extracted and purified under native conditions using Talon metal affinity chromatography according to the protocols recommended by the manufacturer (Clontech). The purified Ep85 was used for different assays and to raise anti-Etk antibody in rabbits.

Bacterial cell fractionation

EPEC culture (200 ml) was grown to a density of OD = 1.0 in LB, 37°C. The culture was harvested, washed, resuspended in 1 ml of cold sonication buffer [10 mM Tris-HCl pH 7.5, 0.4 mM VO_4Na_3 , 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF) and 10 µg/ml leupeptin] and sonicated. Cell envelopes and unbroken bacteria were removed by centrifuging twice (5000 g for 5 min). The cleared supernatant containing cytosolic and periplasmic soluble proteins and inner and outer membranes was removed to fresh tubes and further centrifuged for 1 h at 50 000 g, 4°C to pellet the membranes. The supernatant containing soluble proteins was removed (S-fraction), and the membrane pellet was washed with sonication buffer and resuspended in 0.1 ml of Sarkosyl buffer [100 mM NaCl, 10 mM Tris-HCl pH 8.0, 0.4 mM VO_4Na_3 , 0.1 mg/ml PMSF, 10 µg/ml leupeptin and 0.5% *N*-lauroylsarcosine (Sigma)]. Under these conditions, the inner membrane is dissolved but not the outer membrane (Nikaido, 1994). The outer membranes were precipitated by centrifugation (50 000 g, 1 h) and the supernatant containing the solubilized inner membrane proteins was collected (I-fraction). The outer membrane pellet was washed in Sarkosyl buffer, precipitated again by centrifugation as before, and dissolved in 0.1 ml of SDS loading buffer (O-fraction). Appropriate amounts of 5× SDS loading buffer were added to the different fractions before subjecting them to SDS-PAGE.

Dephosphorylation with YopH

The purified tyrosine-phosphorylated Etk was treated with 5 U of the specific tyrosine protein phosphatase YopH, according to the manufacturer's recommendations (BioLabs). The reaction was stopped at 30, 60 and 90 s after adding YopH by removing 20 µl aliquots of the reaction mixture into tubes containing 1 µl of 100 mM Na_3VO_4 . Then 5 µl of each aliquot was subjected for immunoblot analysis.

Protein kinase assays

Autophosphorylation was performed by adding 5 µl of purified Etk (~1 µg of Etk/µl) into 190 µl of reaction mix (150 mM NaCl, 10 mM MgCl_2 , 10 mM Tris-HCl, pH 7.4). The reaction was started by adding 5 µl of ATP (final concentration of 25 µM ATP, and 10 µCi of

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$). The reaction was stopped by removing 20 µl aliquots at different time points into tubes containing 5 µl of 500 mM EDTA. Then 5 µl of each aliquot was used for analysis by SDS-PAGE. Phosphorylation of poly(Glu:Tyr) (4:1, Sigma) was carried out in 40 µl containing 10 mM MgCl_2 , 20 mM Tris-HCl pH 7.5, 20 µM ATP, 1 µCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, 10 µl of suspended Talon beads with bound Etk (~1 µg of Etk/µl) and poly(Glu:Tyr) at the indicated concentration. The reaction was stopped by short centrifugation and application of 25 µl of the Etk-free supernatant onto Whatman 3MM paper which was then washed three times with 10% trichloroacetic acid and once with ethanol, dried and used to count radioactivity. In all experiments, we included control with no enzyme (Etk) to exclude non-specific association of ATP with poly(Glu:Tyr). To ensure that poly(Glu:Tyr) is the substrate, we included control without exogenic substrate [i.e. poly(Glu:Tyr)]. In this case, only low levels of labelling were detected (~400 c.p.m.) which represent low levels of Etk leakage from the Talon beads. As an additional control, we analysed the reaction by SDS-PAGE and autoradiography. The phosphorylated poly(Glu:Tyr) appears as a distinct smear 20–60 kDa in size (data not shown).

Phosphoamino acid analysis

This analysis was carried out as described by Duclos *et al.* (1991). Briefly, the purified Etk was labelled *in vitro* with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described above and hydrolysed in 200 µl of 6 M HCl for 2 h at 110°C. The hydrolysate was dried in a Speed-Vac concentrator and resuspended in 20 µl of water containing 1 mg/ml of each of the phosphoamino acid markers P-Ser, P-Thr and P-Tyr (Sigma). Two microlitres of the hydrolysate were analysed by ascending thin-layer chromatography (TLC) cellulose, Merck Inc.) using a solvent containing a mix of isobutyric acid and 0.5 M NH_4OH (5:3, v/v). The position of phosphoamino acid markers (P-Ser, P-Thr and P-Tyr) was detected by ninhydrin staining of the TLC plate (0.25% ninhydrin in acetone). The plate was then exposed to X-ray film to locate the position of the ^{32}P -labelled amino acids.

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