Editor's Choice

Pseudomonas aeruginosa injects NDK into host cells through a type III secretion system

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Pseudomonas aeruginosa is a Gram-negative opportunistic human pathogen possessing a type III secretion system (T3SS) which injects toxic effector proteins into mammalian host cells. In previous studies, *P. aeruginosa* strains lacking all of the known type III effectors were shown to cause cytotoxicity upon prolonged infection time. In this study, we report the identification of a new cytotoxin, nucleoside diphosphate kinase (NDK), which is injected into eukaryotic cells in a T3SS-dependent manner. Injection of NDK is inhibited by the presence of previously known effectors of the T3SS, with an effectorless strain injecting the highest amount, suggesting active competition with the known T3SS effectors. NDK is shown to cause a cytotoxic response when expressed in eukaryotic cells, and *P. aeruginosa* strains harbouring NDK also show a greater toxicity than strains lacking it. Interestingly, the cytotoxic effect of intracellular NDK is independent of its kinase activity. In previous studies, NDK was shown to be secreted into culture supernatants via a type I secretion system and cause cytotoxicity in a kinase-dependent manner. Therefore, the current study highlights an alternative route of NDK secretion as well as two different cytotoxic mechanisms of NDK, depending on the extra- or intra-cellular location of the protein.

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen responsible for causing diseases in immunocompromised individuals, most notably those suffering from severe burns or cystic fibrosis (Lyczak *et al.*, 2000). In order to maintain infection, *P. aeruginosa* relies on the production of numerous virulence factors, some of which are injected directly into host cells via a cell contact-mediated type III secretion system (T3SS) (Galán & Collmer, 1999; Cornelis & Van Gijsegem, 2000). The T3SS is a proteinaeous needle which translocates proteins, known as effectors, directly from the bacterial cytoplasm into the host cell (Hayes *et al.*, 2010). The effectors secreted by the T3SS of *P. aeruginosa* – exoenzymes S, T, Y and U – are the major contributors to acute toxicity during the course of an infection (Barbieri & Sun, 2004; Engel & Balachandran, 2009; Hauser, 2009). The majority of *P. aeruginosa* isolates encode three of the four type III effectors, either STY or UTY (Feltman *et al.*, 2001).

We have recently demonstrated that *P. aeruginosa* could be utilized to deliver functional nuclear proteins into pluripotent and differentiated cells by using a laboratory strain, known as PAK-J, which displays an elevated secretion of effectors compared to most previously characterized strains of *P. aeruginosa* (Bichsel *et al.*, 2011, 2013). The strain used to deliver nuclear proteins, PAK-JΔSTY, was devoid of all three known type III secreted exotoxins so as to reduce cytotoxicity to the host cells. Despite the depletion of the type III secreted effectors, cytotoxic effects were still observed in cells after prolonged exposure to the bacteria. In contrast, mutants that are defective in the T3SS display almost no cytotoxicity, suggesting the presence of additional effectors secreted by the T3SS.

In an effort to identify the additional type III secreted effectors, we found that nucleoside diphosphate kinase (NDK) is injected into HeLa cells by a strain lacking all T3SS effectors, including ExoS, ExoT and ExoY. NDK is an ATP-utilizing enzyme that is secreted by the type I secretion system (T1SS) in *P. aeruginosa* and had previously been shown to cause cytotoxicity when incubated with macrophages (Zaborina *et al.*, 1999; Kamath *et al.*, 2000). In this report, we demonstrate that NDK is translocated into host cells in a T3SS-dependent manner and that intracellular NDK causes HeLa cell lifting independent of its kinase

activity. This was further proven by transfection of a plasmid expressing a kinase-defective NDK in HeLa cells. Results from the current study suggest a new mechanism of cytotoxicity for the NDK protein during host–*Pseudomonas* interactions.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. Strain PAK-J is a laboratory strain of *P. aeruginosa* while PAK-JΔ*STYN* is deleted of the three type III effectors (*exoS*, *exoT* & *exoY*) as well as a negative regulator of T3SS (*popN*). Strain PAK-JΔ*STYN* was further deleted of the type II secretion system (*xcpQ*) and quorum sensing (*lasI* & *rhlI*) to result in PAK-JΔ7. Strain PAK-JΔ8 is an *ndk* deletion derivative of the PAK-JΔ7. Both *E. coli* and *P. aeruginosa* were grown in Luria (L) broth or on L agar plates at 37 °C. For plasmid selection, the final concentration of antibiotics used was 150 μg carbenicillin ml^{-1} for *P. aeruginosa* and 100 μg of ampicillin ml^{-1} for *E. coli*. Primers used to generate various constructs are listed in Table 2. Human and *E. coli* *ndk* genes were PCR amplified and cloned into the pUCP20 vector using *Bam*HI and *Hin*dIII restriction sites located at the end of each primer. *P. aeruginosa* *ndk* was cloned into pUCP20 using *Eco*RI and *Pst*I restriction sites and was further cloned into the eukaryotic expression vector pCDNA3.1(+) using *Bam*HI and *Eco*RI sites.

Cell culture. HeLa and H1299 cells were cultured in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10 % FBS and 1 % penicillin/streptomycin (Gibco). Cells were incubated at 37 °C with 5 % CO$_2$.

Protein secretion assay. Bacterial strains were grown overnight in 1.0 ml of L broth containing carbenicillin at 37 °C. Overnight cultures were then inoculated at 5 % into fresh L broth containing antibiotics for non-type III inducing conditions and L broth plus antibiotics and 5 mM EGTA for type III inducing conditions. *P. aeruginosa* strains were grown in a shaking incubator at 37 °C for 3 h, after which bacterial cells were centrifuged at 20 000 *g* for 2 min. Bacterial supernatants were collected, precipitated by 15 % TCA (20× concentration), resuspended in 1× SDS protein sample buffer and boiled for 10 min before SDS-PAGE analysis.

Protein injection assay. HeLa cells were seeded onto six-well plates at approximately 70 % confluence (8.4×10^5 cells) in DMEM containing 10 % FBS, penicillin and streptomycin (pen/strep) the night before infection. Two hours prior to infection, cells were washed twice in PBS and replaced with DMEM+10 %FBS without pen/strep. Bacterial strains were grown in L broth supplemented with carbenicillin at 37 °C until the OD$_{600}$ reached 0.8. For an m.o.i. of 20, 2×10^7 c.f.u. were incubated with HeLa cells. Following infection, culture medium was removed and the HeLa cells were washed three times with PBS, followed by trypsinization. HeLa cells were centrifuged at 500 *g* for 5 min and then washed three times with PBS. HeLa cell pellets were suspended in 40 μl of PBS containing 0.25 % Triton X-100 and placed on ice for 10 min, which selectively lyses HeLa cells (Ha & Jin, 2001). Cell lysates were then centrifuged at 20 000 *g* for 2 min and the supernatants were mixed with an equal volume of 2× SDS protein sample buffer. Samples were boiled for 10 min and stored at −20 °C until use.

Western blotting. Secretion and injection samples were loaded and separated on 4–20 % gradient SDS-PAGE gels (Bio-Rad). Proteins

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source
P. aeruginosa		
PAK-J	PAK derivative with enhanced T3SS	Bichsel *et al.* (2011)
PAK-JΔ*S*	PAK derivative with chromosomal deletion *exoS*	Bichsel *et al.* (2011)
PAK-JΔ*T*	PAK derivative with chromosomal deletion *exoT*	Bichsel *et al.* (2011)
PAK-JΔ*ST*	PAK derivative with chromosomal deletion of *exoS* and *exoT*	Bichsel *et al.* (2011)
PAK-JΔ*STY*	PAK derivative with chromosomal deletion of *exoS*, *exoT* and *exoY*	Bichsel *et al.* (2011)
PAK-JΔ*STYN*	PAK-JΔ*STY* with chromosomal deletion of *popN*	This study
PAK-JΔ*pscF*	PAK derivative with chromosomal deletion of *pscF*	This study
PAK-JΔ7	PAK-JΔ*STYN* with chromosomal deletion of *xcpQ*, *lasR-I* and *rhlR-I*	This study
PAK-JΔ8	PAK-JΔ7 with chromosomal deletion of *ndk*	This study
Plasmids		
pUCP20	*Escherichia–Pseudomonas* shuttle vector; Ap^{r}	West *et al.* (1994)
pPaNDK	FLAG-tagged *ndk* from *P. aeruginosa* in pUCP20; Ap^{r}	This study
pPaNDKH117Q	pPaNDK with kinase null *ndk* mutant; Ap^{r}	This study
pPaNDKH117QH54Q	pPaNDKH117Q with additional H54Q mutation; Ap^{r}	This study
pEcNDK	FLAG-tagged *ndk* from *E. coli* in pUCP20; Ap^{r}	This study
pHuNDK	FLAG-tagged *ndk* from human origin in pUCP20; Ap^{r}	This study
pCDNA3.1(+)	Eukaryotic expression vector containing CMV promoter; Ap^{r}	Invitrogen
pJJ0322	*exoS* in pEGFP-C1; Km^{r}	Jia *et al.* (2006)
pDNNDK	*ndk* from *P. aeruginosa* in pCDNA3.1(+); Ap^{r}	This study
pDNNDKH117Q	pDNNDK with kinase null *ndk* mutant; Ap^{r}	This study
pEGFP-1	Mammalian expression vector with CMV promoter driven *egfp*; Km^{r}	BD Clontech
pNDKΔ8	NDK in pPaNDK deleted of C-terminal 8 aa; Ap^{r}	This study
pCre-NDK	Full-length NDK fusion to Cre in pUCP20; Ap^{r}	This study
pCre-NDKΔ8	NDKΔ8 fused to Cre in pUCP20; Ap^{r}	This study
pExoS54-Cre	ExoS54 fused to Cre in pUCP20; Ap^{r}	Bichsel *et al.* (2011)

Table 2. List of primers

Construct	Primer
NDK FLAG constructs	
PaNDK upstream	5′-ggagaattcGCGCCTGGCCATCGCGGCGCAGATGG-3′
PaNDK downstream	5′-GGACTGCAGTCACTTGTCGTCATCGTCCTTGTAGTCGCGAATGCGCTCGCAGACTTCGGTAGCCGC-3′
EcNDK upstream	5′-ACCGGATCCCGCGACAGTGAAATTTGTCATGCAATAGTC-3′
EcNDK downstream	5′-ACCAAGCTTTCACTTGTCGTCATCGTCCTTGTAGTCACGGGTGCGCGGGCACACTTCGCCTTC-3′
Human NDK upstream	5′-ACCGGATCCCGCGACAGTGAAATTTGTCATGCAATAGTC-3′
Human NDK downstream	5′-ACCAAGCTTTCACTTGTCGTCATCGTCCTTGTAGTCTTCATAGACCCAGTCATGAGCACAAGA-3′
PaNDK pCDNA3.1 UP	5′-ACCGGATCCGCCATGGCACTGCAACGCACCCTGTCCATCATC-3′
PaNDK pCDNA3.1 DN	5′-ACCGAATTCTCAGCGAATGCGCTCGCAGACTTCGGTAGCCGC-3′
NDKΔ8 downstream	5′-GGAGAGCTCTCACTTGTCGTCATCGTCCTTGTAGTCGAAGAAGTAGGCGATCTCGCGAGCGG-3′
NDK mutations	
NDKH117Q Forward	5′-CGAGAACGCCGTCCAGGGATCCGATTCCGAAGCTTCC-3′
NDKH117Q Reverse	5′-GGAAGCTTCGGAATCGGATCCCTGGACGGCGTTCTCG-3′
NDKH54Q Forward	5′-GGCTTCTATGCCGAGCAGAAAGAGCGTCCGTTCTTC-3′
NDKH54Q Reverse	5′-GAAGAACGGACGCTCTTTCTGCTCGGCATAGAAGCC-3′

were transferred onto PVDF membranes and subjected to immuno-blotting using an anti-FLAG antibody (mouse M2 monoclonal Ab; Sigma) for NDK and anti-β-actin (Santa Cruz) for actin, with a 1000-fold dilution.

HeLa cell lifting assay. HeLa cells were infected with *P. aeruginosa* at the indicated m.o.i. for indicated periods of time using procedures described above. Following infection, HeLa cells were washed three times with PBS to remove non-adhering cells, and then the adhering cells were suspended by incubation with 0.25 % trypsin for 5 min. The number of cells in suspension was counted using a haemocytometer.

Assay for type III injected Cre–NDK fusion protein. To assess the DNA recombinase activity of the injected Cre-NDK fusion protein, a cell line (TE26) with a *lacZ* reporter gene was infected with *P. aeruginosa* strain PAK-JΔSTY harbouring the Cre–NDK fusion construct at an m.o.i. of 50 for 3 h. The TE26 cells were washed with PBS three times and grown for 48 h on DMEM + 10 % FBS with 10 μg ciprofloxacin ml^{-1}. The cells were then subjected to staining for β-galactosidase activity as described previously (Bichsel *et al.*, 2013).

RESULTS

Strains lacking known type III secreted effectors are still cytotoxic

Our previous studies have demonstrated that *P. aeruginosa* is capable of delivering functional Cre recombinase and MyoD into the host cell nucleus when they were fused to the secretion signal sequence of the type III effector ExoS (Bichsel *et al.*, 2011, 2013). We generated a strain PAK-JΔSTY, devoid of all three known type III secreted effectors, to use as the protein delivery strain. Although this strain shows reduced cytotoxicity when incubated with mammalian cells for less than 3 h, observations from longer infection times revealed that this strain possesses residual toxicity. To determine the degree of adverse effects resulting from longer incubation with PAK-JΔSTY, we compared the number of HeLa cells that remained adhered to tissue culture plates following infection by the wild-type strain PAK-J or PAK-JΔSTY. For *P. aeruginosa* mediated cytotoxicity, a high correlation between the results of the HeLa cell lifting assay and that of the LDH release assay had previously been observed (Bichsel *et al.*, 2011). Fig. 1(a) shows that at 4 h post-infection at an m.o.i. of 50, 50 % of the cells remained adhered to the plate following incubation with PAK-JΔSTY whereas incubation with the wild-type strain resulted in only 5 % of cells still adhering. These results are in agreement with previous cytotoxicity studies, which showed that *P. aeruginosa* strains lacking type III effectors still caused low levels of LDH release from cells following infection (Lee *et al.*, 2005; Vance *et al.*, 2005; Bichsel *et al.*, 2011). To address whether the residual HeLa cell lifting effect related to the T3SS, a type III defective mutant PAKΔpscF (defective of the type III needle subunit) was also incubated with HeLa cells. PAKΔpscF displayed a dramatically reduced HeLa cell lifting capacity, as there was only a 10 % reduction in the number of adhering cells compared to a non-infected control (Fig. 1a). These results suggested that the T3SS of *P. aeruginosa*, even in the absence of all known effectors, is still capable of causing toxicity.

Thus far, only four type III exotoxins have been identified among *P. aeruginosa* isolates, and the majority of isolates possess three of the four (Feltman *et al.*, 2001). Therefore, it is possible that additional minor proteins are secreted through the type III needle, especially in the absence of the major effectors. To investigate this possibility, we compared secreted protein patterns of PAK-J (wild-type) and PAK-JΔSTYN (a mutant strain lacking all three known type III secreted effectors and *popN*), following growth under T3SS inducing conditions. The PopN protein is a regulatory protein whose gene deletion results in a strain that constitutively secretes effector proteins (Sundin *et al.*, 2004; Yang *et al.*, 2007). Bacterial strains were grown in L broth in the presence of 5 mM EGTA for 3 h at 37 °C (a standard type III inducing condition) and the supernatants were TCA precipitated, subjected to SDS-PAGE, and stained with
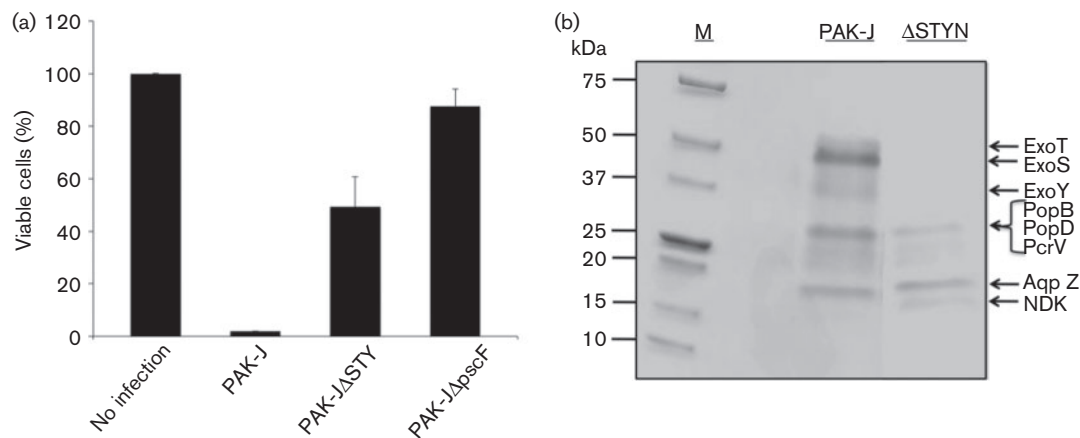
Fig. 1. Strains lacking type III effectors are cytotoxic. (a) Toxicity assay of various *P. aeruginosa* strains. HeLa cells were infected with the indicated strains for 4 h at an m.o.i. of 50, and cells that remained adhered were counted. PAK-J, wild-type; PAK-J*ΔSTY*, deleted of all three type III secreted toxins; PAKΔ*pscF*, T3SS defective mutant. Data represent means of three replicate experiments. Error bars represent SD. (b) Type III protein secretion profiles of wild-type PAK-J and PAK-J*ΔSTYN* mutant. Bacteria were grown in LB with 5 mM EGTA for 3 h, and culture supernatants were concentrated and subjected to Coomassie blue staining.

Coomassie blue. As shown in Fig. 1(b), wild-type PAK-J secretes ExoS, T and Y, while strain PAK-J*ΔSTYN* lacks all these effectors as expected. Mass spectrometric analysis of the two bands shared by the two strains indicated a mixture of translocon proteins (PopB, PopD and PcrV) and a water channel protein aquaporin Z (AqpZ) (Fig. 1b). Most interestingly, strain PAK-J*ΔSTYN* secretes an additional small protein about 15 kDa in size, which was identified as a nucleoside diphosphate kinase (NDK). This kinase is known to convert nucleoside diphosphates (NDPs) to nucleotide triphosphates (NTPs) and has previously been shown to be secreted through a *P. aeruginosa* T1SS (Kamath *et al.*, 2000; Spooner & Yilmaz, 2012).

PAK-J secretes and injects NDK into eukaryotic cells

Based on the results shown in Fig. 1(b), we set out to determine if the T3SS plays a role in the secretion of NDK and assess whether the known type III effectors have any role in this. To accomplish this, a plasmid containing a C-terminal FLAG-tagged form of the NDK from *P. aeruginosa* (pPaNDK) was introduced into various exotoxin deletion derivatives of PAK-J. Protein secretion was determined following bacterial growth in L broth or L broth containing 5 mM EGTA for 3 h at 37 °C. Bacterial culture supernatants were collected, separated on SDS-polyacrylamide gel, and subjected to immunoblotting using an antibody against FLAG tag. As the results shown in Fig. 2(a) all of the strains secreted similar amounts of NDK under type III inducing or non-inducing conditions, suggesting that the T3SS may not play a role in the secretion of the protein. The fact that the type III defective mutant, PAKΔ*pscF*, secretes NDK at amounts similar to strains possessing a functional T3SS further suggests that the T3SS may not be

involved in the observed NDK secretion. Additionally, deletion of known type III effectors does not appear to have any effect on the secretion of NDK, as all of the strains secreted NDK at similar levels.

Although the secretion data shown in Fig. 2(a) suggest that the T3SS does not play a role in the secretion of NDK, it was possible that the amount of NDK secreted by the T3SS was so low that no detectable differences could be observed between type I and III secreted proteins in the above assay. If NDK is injected through the type III injectisome, then it should behave like other type III effectors and be injected into eukaryotic cells. We therefore incubated the same deletion strains harbouring FLAG-tagged NDK with HeLa cells for 3 h at an m.o.i. of 20. Following the infection, HeLa cells were lysed and the cell lysates were subjected to immunoblotting with an anti-FLAG antibody. Fig. 2(b) shows that PAK-J*ΔSTY*/pPaNDK injects the highest amount of NDK into HeLa cells and that the amount injected decreased as one or more known type III effector genes were present, with almost none being injected from the wild-type PAK-J strain. Similar results were also obtained when another cell line, human non-small cell lung carcinoma, H1299, was used in place of the HeLa cells (Fig. 2b). Since the amount of NDK injection was negatively influenced by the presence of known type III effectors, the results suggested that NDK might also be injected through T3SS and be competitively inhibited by other effectors. Indeed, infection with the T3SS defective strain (PAKΔ*pscF*) showed no NDK injection, indicating a functional T3SS is required for NDK translocation. Further, no intracellular NDK was detectable following 3 h of incubation of HeLa cells with the culture supernatant of strain PAK-J*ΔSTY*/pPaNDK (data not shown), suggesting that the NDK signals in Fig. 2(b) are the result of T3SS-dependent translocation and not a direct uptake of NDK by the HeLa cells from the culture
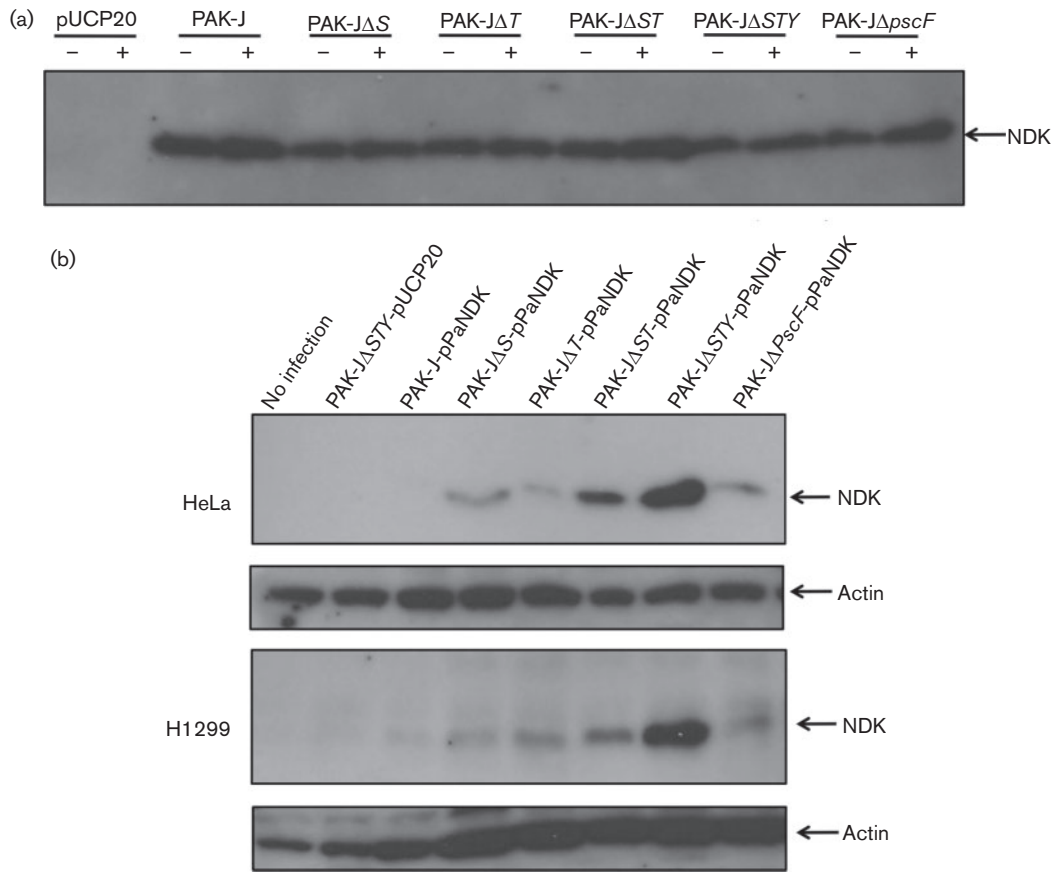
Fig. 2. Secretion and injection of an NDK–FLAG construct. (a) Detection of NDK–FLAG in the culture supernatant of *P. aeruginosa*. Bacteria containing pPaNDK were grown under type III inducing (+) and non-inducing (−) conditions, and culture supernatants were directly subjected to Western blotting using an anti-FLAG antibody. (b) HeLa and H1299 cell lysates that were infected with the indicated bacterial strains were subjected to Western blotting using an anti-FLAG antibody. Membranes were stripped and reprobed with an actin antibody to serve as a loading control.

supernatant. Taken together, the above results suggested that NDK is secreted into the culture supernatant mainly through the T1SS, while a small amount is injected into host cells via a functional T3SS. Also, a higher amount of NDK injection into HeLa cells was observed in bacteria that lack known type III effectors, suggesting that these effectors may compete with NDK for injection through the type III secretion machinery.

Intracellular NDK is cytotoxic to HeLa cells

NDK has been reported to cause cytotoxicity in macrophages by disrupting extracellular ATP concentrations (Zaborina *et al.*, 1999); however, it has not been determined whether bacterial NDK is capable of eliciting a cytotoxic response when expressed inside host cells. To examine this, NDK was cloned into a eukaryotic expression vector pCDNA3.1 and co-transfected into HeLa cells with a plasmid expressing GFP (pEGFP). If NDK is toxic to the host cell, the number of GFP positive cells should be lower in samples co-transfected with pEGFP and NDK, compared to

cells co-transfected with pEGFP and pCDNA3.1 (empty vector control). Forty-eight hours post-transfection, HeLa cells were collected and analysed by flow cytometry to quantify the number of GFP positive cells. Fig. 3(a) shows transfection with GFP and empty vector (pEGFP + pCDNA3.1 +) resulted in 38 % of HeLa cells expressing GFP, whereas co-transfection of GFP and NDK (pEGFP + pDNNDK) resulted in a 12 % reduction in the amount of GFP positive cells compared with the GFP/empty vector transfected cells. The pEGFP was also co-transfected with a plasmid expressing the acute type III secreted cytotoxin ExoS (pJJ0322), resulting in an 80 % decrease in GFP positive cells compared with the GFP/empty vector transfected cells (Fig. 3b). Although NDK caused a significant reduction in the amount of GFP positive cells, the toxicity was not as robust as ExoS. This would suggest that NDK is not an acute toxin, but in the absence of other type III effectors, it does play a significant role in toxicity.

To further assess NDK-mediated cytotoxicity, we deleted NDK via homologous recombination in a laboratory strain PAK-JΔ7, resulting in a strain PAK-JΔ8. Strain PAK-JΔ7 is
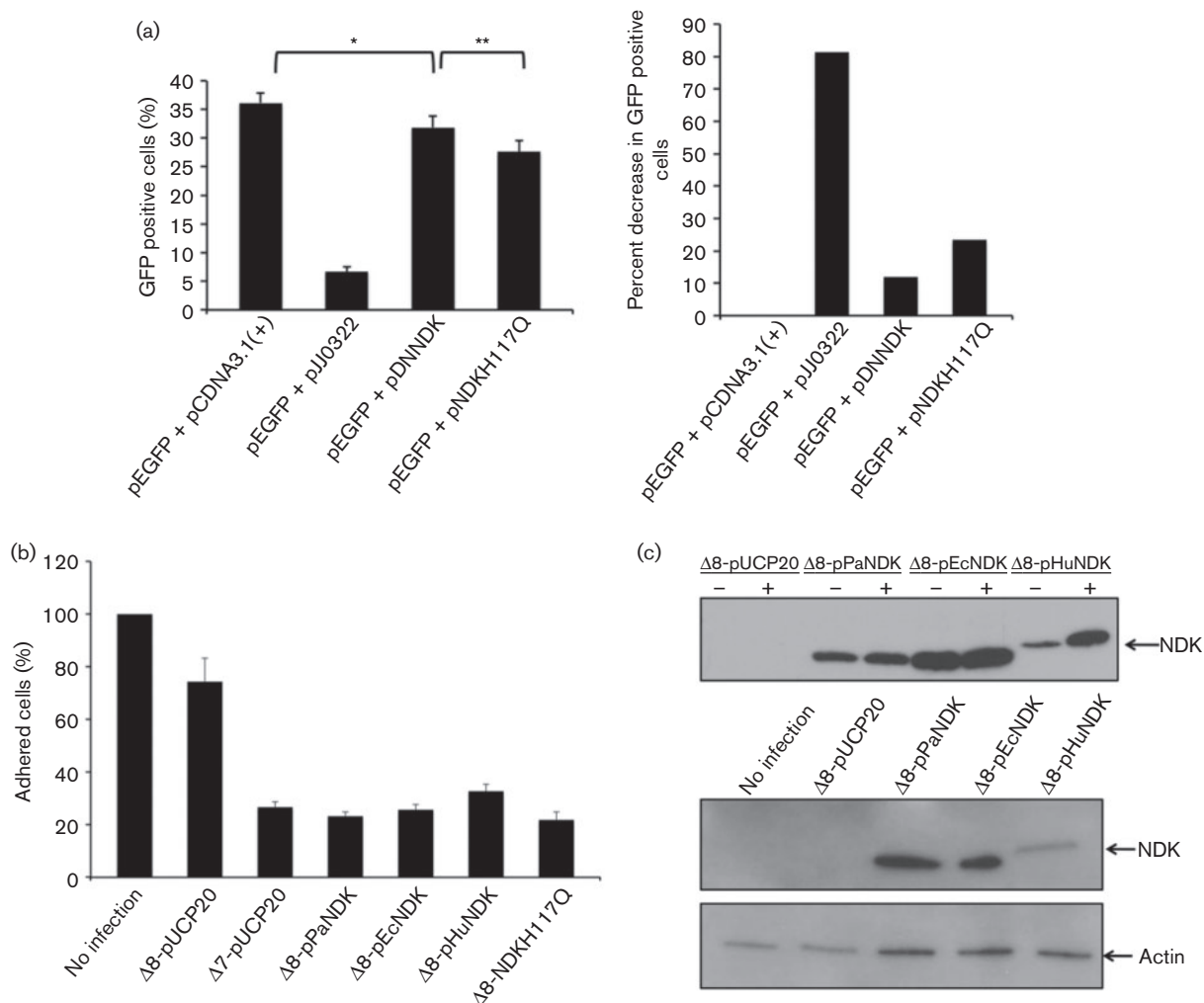
Fig. 3. Cytotoxicity of strains containing NDK. (a) Cytotoxicity resulting from the expression of NDK in a eukaryotic expression vector. Plasmids containing GFP and NDK were co-transfected into HeLa cells. After 48 h, the number of GFP positive cells was quantified using flow cytometry. Data represent means of three independent replicates. Error bars represent SD. *$P=0.017$ and **$P=0.023$ using Student's t-test. (b) Cytotoxicity from strains containing NDK. Bacteria were incubated for 5 h at an m.o.i. of 500 and cells remaining adhered were collected and counted. Data from three independent replicates. (c) Secretion and injection assay with *P. aeruginosa* expressing various *ndk* genes. Bacteria were grown under type III inducing and non-inducing conditions and culture supernatants were subjected to Western blotting using an anti-FLAG antibody. HeLa cells were infected at an m.o.i. 20 for 3 h, selectively lysed and subjected to Western blotting with an anti-FLAG antibody.

a derivative of PAK-J∆*STYN*, with additional deletion of the type II secretion system and quorum sensing (Table 1) in an effort to further reduce the bacterial toxicity. Although this strain had the major virulence factors removed, it still shows cytoxicity when incubated with mammalian cells. To determine if NDK expressing strains possess greater toxicity, HeLa cells were infected with PAK-J∆7, PAK-J∆8, or PAK-J∆8 complemented with an *ndk* expressing plasmid (pPaNDK). Free-floating bacterial cells were then washed off with PBS and fresh media containing ciprofloxacin were applied to the cells to eliminate residual bacteria. Twenty-four hours later, lifted HeLa cells were washed away and the cells that remained adhered were trypsinized and counted using a haemocytometer. As shown

in Fig. 3(b), incubation with PAK-J∆7 at an m.o.i. of 100 for 5 h resulted in a 75 % reduction in the number of adhered cells compared with the uninfected control. However, incubation with PAK-J∆8 resulted in only a 25 % decrease in adhered cells. Complementation of PAK-J∆8 with a wild-type *ndk* gene restored toxicity, causing a similar amount of cell lifting to infection with PAK-J∆7. NDK containing strains do elicit a toxic response; however, the toxicity is not as robust, and the time necessary to cause toxicity is much longer compared with the well-known type III secreted effector ExoS.

Previous reports have demonstrated that NDK from *Mycobacterium tuberculosis* is also responsible for causing

toxicity when incubated with eukaryotic cells (Chopra *et al.*, 2003, 2004). We were curious as to whether this was a general feature of NDKs or relevant only to *P. aeruginosa* and *M. tuberculosis*. To examine this, NDKs from *E. coli* (pEcNDK) and humans (pHuNDK) were cloned into pUCP20 and introduced into *P. aeruginosa*. NDK of *P. aeruginosa* shares 60 % amino acid homology with that of *E. coli* and 42 % with that of humans (Chakrabarty, 1998). As shown in Fig. 3(c), NDKs from *E. coli* and human are also secreted into the culture supernatant by *P. aeruginosa*. Testing these strains for protein injection revealed that NDK from *E. coli* was injected at amounts similar to that from *P. aeruginosa*, whereas the human form showed a significantly lower amount of injection (Fig. 3c). The toxicity assay results in Fig. 3(b) reveal that strains possessing *E. coli* and human NDKs also cause HeLa cells to detach from the monolayer, with 75 % detaching with *E. coli* NDK and roughly 65 % with the human form. Interestingly, the human form of NDK is secreted at levels similar to the bacterial NDKs; however, the amount injected was much lower, possibly due to the lack of the signal necessary for injection via the type III needle. Although the human NDK causes a cytotoxic response, it is slightly reduced compared with that from *E. coli* or *P. aeruginosa*, which may relate to the observation that human NDK is not as readily injected. Based on these observations, it seems that a relatively low amount of injected NDK is sufficient to elicit cytotoxic response. Taken together, these results suggest that NDKs from *E. coli* and humans are able to generate a cytotoxic response when injected into eukaryotic cells.

Kinase activity of NDK is not required for its toxicity

NDK is responsible for generating nucleotide triphosphates (NTPs) from nuclotide diphosphates (NDPs) by transfer of a terminal phosphate from an NTP to an NDP (Spooner & Yilmaz, 2012). To date, all known prokaryotic and eukaryotic NDKs possess a conserved histidine residue (H117) that becomes phosphorylated during the generation of NTPs (Chakrabarty, 1998). Mutation of H117 to either A or Q has previously been shown to abolish the kinase activity of NDK (Tiwari *et al.*, 2004). We therefore wanted to determine whether the kinase activity was responsible for the toxicity elicited by the NDK. To accomplish this, the histidine H117 of *P. aeruginosa* NDK was mutated to glutamine (H117Q) through site-directed mutagenesis. As shown in Fig. 3(a), co-transfection of the mutant NDK (pNDKH117Q) with GFP resulted in an 18 % reduction in the amount of GFP positive cells when compared with co-transfection of GFP/empty vector control. Surprisingly, the toxicity was slightly greater in the mutant than in the wild-type, suggesting that the kinase activity is not linked to the toxicity of NDK. Consistent with this, infection with a *P. aeruginosa* strain harbouring a kinase-null NDK resulted in a toxic effect similar to that of wild-type NDK containing strains (Fig. 3b). These assay results suggest that the

toxicity of NDK in mammalian cells is independent of its kinase activity.

There is another conserved histidine in NDK at position 54. We further mutated this site in the background of the NDK (H117Q) mutant. The strain PAK-JΔ8 harbouring the resulting double point mutant NDK caused a similar HeLa cell lifting profile to the single H117Q mutant (data not shown), eliminating the possible involvement of secondary kinase domain causing the toxic effect.

C-terminal peptide of NDK is essential for injection via a functional T3SS

A truncated version of the NDK, lacking the C-terminal 8 aa (Fig. 4a) was generated, which have previously been shown to be essential for the T1SS-dependent NDK secretion (Kamath *et al.*, 2000). The construct was then introduced into the PAK-JΔ8 strain and tested for secretion under type III inducing conditions as well as injection into HeLa cells. Independent of type III inducing conditions, the full-length NDK was secreted into the extracellular media, whereas the truncated form was not detectable in the culture supernatant, although similar levels of protein were produced inside the bacterial cells (Fig. 4b). These results are in agreement with a previous report which demonstrated that NDK secretion through the T1SS is dependent on a DTEV motif located in the last 8 aa of the carboxyl terminus (Kamath *et al.*, 2000). Interestingly, the truncated form of NDK appears to run at a higher molecular mass than the full-length form, although sequencing results confirmed the gene sequence was correct, so the discrepancy may represent a post-translational modification. The NDK of *P. aeruginosa* has previously been shown to be cleaved from 16 to 12 kDa by elastase (Kamath *et al.*, 1998). It is possible that the C-terminal deletion represents the uncleaved 16 kDa band, implicating the requirement of C-terminal peptide for elastase mediated proteolytic processing.

To determine if the C-terminal peptide of NDK is essential for injection via the T3SS, bacterial strains containing the two forms of NDK were incubated with HeLa cells at an m.o.i. of 20 for 3 h. As shown in Fig. 4(c), the full-length NDK was readily translocated into HeLa cells while the truncated protein was not detectable, suggesting it is not readily injected into the host cells. The above data suggested that the C-terminal 8 aa are required for both T1SS-dependent secretion and T3SS-dependent injection of NDK. Whether the C-terminal 8 aa function as a secretion signal for both the T1SS and the T3SS, or whether T1SS-dependent secretion is a prerequisite for T3SS-dependent injection is not clear at the present time.

To further confirm the above observations, an alternative protein injection assay system was utilized. We have previously developed an assay system where T3SS-dependent injection of Cre recombinase can efficiently cause LoxP mediated recombination on the host chromosome, leading
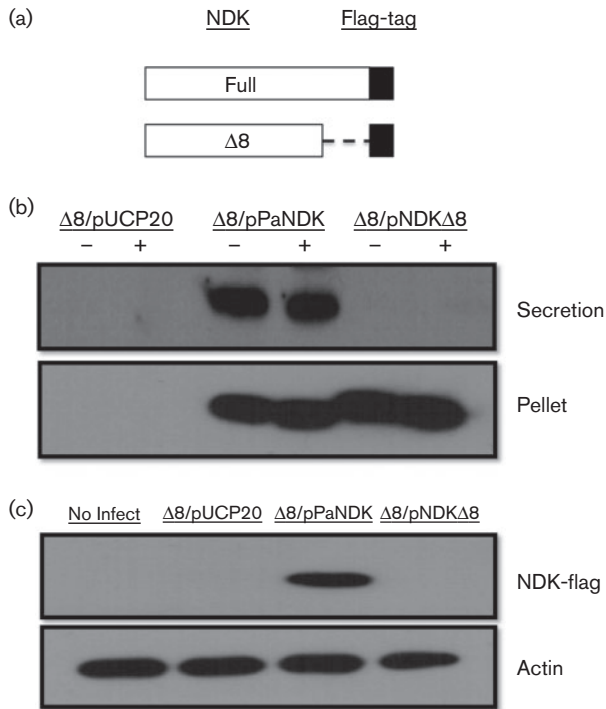
Fig. 4. Injection and secretion of truncated NDK. (a) A truncated form of NDK, lacking the last 8 aa, was fused with a FLAG-tag. (b) Detection of truncated NDK from bacterial culture supernatants. Bacteria were grown under type III inducing and non-induction conditions and bacterial culture supernatants were collected for Western blot. (c) Detection of truncated NDK in HeLa cell lysates. Bacteria were incubated with HeLa cells at an m.o.i. of 20 for 3 h, lysed and probed with an antibody against the FLAG-tag. Membrane was stripped and reprobed with actin as a loading control.

to activation of *lacZ* reporter gene expression (Bichsel *et al.*, 2011). NDK or C-terminal truncated NDK (NDKΔ8) was fused to a Cre recombinase which contains a nuclear localization sequence. Upon introduction of the resulting plasmid constructs into PAK-JΔSTY, fusion proteins with the expected molecular mass were detectable at similar amounts from respective bacterial cell lysates (Fig. 5a). In the culture supernatants, however, the full-length Cre–NDK fusion was detectable but not the Cre–NDKΔ8 fusion, indicating that NDK is able to direct the secretion of the Cre–NDK fusion protein through the T1SS where the C-terminal 8 aa is essential for the secretion. Upon infection of a reporter TE26 cell line (human sarcoma derivative) which encodes a *lacZ* reporter gene whose expression is blocked by a floxed transcriptional terminator from SV40 (Bichsel *et al.*, 2011), the injected intracellular Cre–NDK was clearly detectable by Western blot, but not that of Cre–NDKΔ8 or Cre–NDK in cells infected with a T3SS defective mutant (PAK-JΔpscF/pCre-NDK) (Fig. 5b). Upon colorimetric straining for LacZ enzyme activity 48 h after the bacterial infection, LacZ positive cells were

detectable in TE26 cells that were infected with PAK-JΔSTY/pCre-NDK but not in those infected with PAK-JΔSTY/pCre-NDKΔ8 or PAK-JΔpscF/pCre-NDK (Fig. 5c), confirming that Cre–NDK is indeed injected via the T3SS into the TE26 cells and triggered the LoxP-dependent recombination. The percentage of LacZ positive cells was about 5 %, compared with 40 % in TE26 cells infected by PAK-JΔSTY/pExoS54-Cre. These results further support the notion that NDK is a less efficiently translocated effector than the ExoS.

DISCUSSION

Previous studies, along with data presented here, clearly show that prolonged exposure to *P. aeruginosa* strains lacking all known type III effectors results in significant cytotoxicity (Vance *et al.*, 2005; Bichsel *et al.*, 2011). This toxicity is dependent on the T3SS, as infection with strains lacking a functional T3SS causes almost no cytotoxicity. In a recent study, we reported that the formation of type III translocon pore by the PopB/PopD also contributes to the cytotoxicity (Galle *et al.*, 2012). Therefore, the residual cytotoxicity of the mutant strain PAK-JΔ8 may represent the translocon pore-mediated cytotoxicity (Fig. 1a). To date, only four type III secreted exotoxins have been characterized in *P. aeruginosa*, which is relatively low in number compared with other T3SS-encoding bacteria, such as *Yersinia*, *Salmonella* and *Shigella* species (Coburn *et al.*, 2007). The goal of this study was to determine if any additional proteins are secreted through the T3SS. Examination of secreted proteins in the culture supernatants from a strain of *P. aeruginosa* lacking all three known type III secreted effectors as well as *popN* (PAK-JΔSTYN) revealed that NDK was secreted at a higher level than that by wild-type when grown under type III inducing conditions. The molecular mechanism by which PopN might influence NDK secretion remains to be determined. It should be noted that previous studies had shown that the expression of *ndk* is under the control of AlgQ and inducible by phosphate starvation while NDK secretion is influenced by levels of Ca^{2+}, Mg^{2+} as well as the κ-chain of casein (Zaborina *et al.*, 1999; Ambrosi *et al.*, 2005).

Although not as toxic as the well-known type III effector ExoS, results presented in this study as well as previous reports strongly support a delayed type of cytotoxicity caused by NDK. Previous studies had shown that NDK is secreted into the culture supernatant, presumably through the T1SS pathway; however, it had never been linked to the T3SS or direct injection into the host cytosol. A number of key experimental data suggest that NDK is injected into the host cells through a bacterial T3SS rather than the secreted NDK being taken up by the eukaryotic cells. First, our results demonstrate that a functional T3SS is necessary for the injection of NDK into eukaryotic cells. Second, direct incubation of HeLa cells with the culture supernatant of *P. aeruginosa* strain PAK-JΔSTY/pPaNDK, which contains T1SS-secreted NDK, did not result in detectable amounts
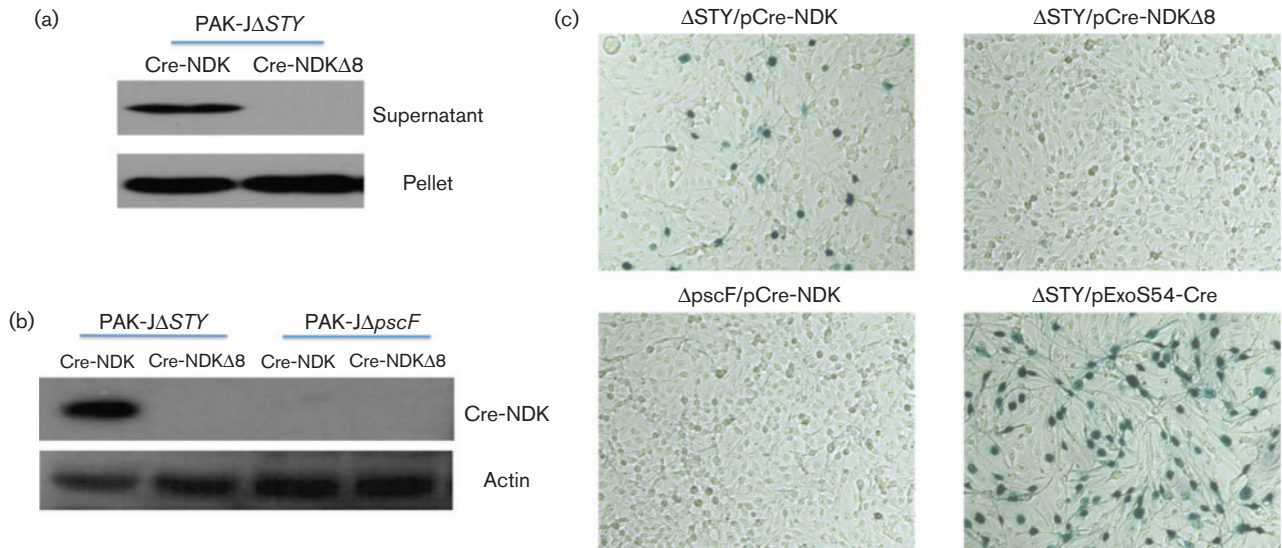
Fig. 5. Injection of Cre–NDK fusions. (a) Detection of the Cre–NDK and Cre–NDKD8 fusions in bacterial cell lysates as well as culture supernatants by anti-Flag Western blot. (b) Detection of Cre–NDK and Cre–NDKD8 fusions in TE26 cell lysates. TE26 cells were incubated with bacterial cells at m.o.i. 20 for 3 h, lysed and probed with anti-Flag Ab. Membrane was stripped and reprobed for actin as a loading control. (c) β-galactosidase staining of the TE26 cells following delivery of the Cre–NDK, Cre–NDKD8 and ExoS54–Cre by *P. aeruginosa* strains PAK-J*DSTY* or Cre–NDK by PAK-J*DpscF*.

of intracellular NDK. Third, the presence of known T3SS effectors inhibits NDK injection, presumably through competition for the T3SS injectisome, also indicating that NDK is a low-affinity substrate for the T3SS.

This is the first report to show that NDK is injected into host cells by the T3SS and that intracellular expression of NDK results in a cytotoxic response. Additionally, toxicity does not appear to be dependent on the kinase activity of this protein, as the kinase defective mutant generates as much toxicity as the wild-type does (Fig. 3a, b). It is therefore possible that NDK from *P. aeruginosa* possesses additional functional domains that are responsible for the observed toxicity. In the case of *M. tuberculosis*, in addition to its kinase activity, NDK has also been shown to possess GAP activity for Rho-GTPases (Chopra *et al.*, 2004; Sun *et al.*, 2010). Considering that extracellular NDK causes cytotoxicity in macrophages by disrupting extracellular ATP through its kinase activity (Zaborina *et al.*, 1999), our current findings suggest that NDK causes cytotoxicity through two completely different mechanisms, depending on whether it is in the extracellular or intracellular space. Further studies are required to understand the mechanism of cytotoxicity of intracellular NDK as well as the biological significance of T3SS mediated NDK injection.

NDK toxicity does not appear to be restricted to that of *P. aeruginosa*, as an NDK defective *P. aeruginosa* complemented by NDK from *E. coli* or human caused similar toxicity (Fig. 3b). Although the human form of NDK is not as readily injected as NDKs from *P. aeruginosa* and *E. coli*, the toxicity was only slightly reduced (Fig. 3c), suggesting

that a small amount of intracellular NDK is sufficient to cause cytotoxicity. Additionally, it is possible that expressing a functional NDK in *P. aeruginosa* results in the activation of additional unknown virulence mechanisms. Recent data from our laboratories show that *P. aeruginosa* strains expressing NDK are able to induce pro-inflammatory cytokine expression in human alveolar epithelium cells (U.-H. Ha, unpublished data). These results were seen in strains expressing NDK from *P. aeruginosa* but not in strains lacking NDK, consistent with the current findings.

NDK does not possess a canonical T3SS signal sequence; however, by making a truncated form of NDK which is defective for T1SS-dependent secretion, it was found that disruption of the type I signal sequence also eliminates injection by the type III needle. A possible explanation for this observation is that the signal sequence for type I secretion overlaps with that for type III secretion. Alternatively, NDK might be secreted into the extracellular milieu first through the T1SS, and then is injected by the type III needle, as such a two-step process is not unprecedented (Vidal & Navarro-García, 2008; Akopyan *et al.*, 2011).

In summary, results from this study have identified NDK as an additional protein injected into eukaryotic cells by the T3SS of *P. aeruginosa*. Although not as toxic as the known type III effectors, in the absence of said effectors, NDK is able to display significant cytotoxicity with prolonged incubation. We have demonstrated that NDK is injected into mammalian cells in a T3SS-dependent manner, causing a cytotoxic effect independent of its kinase

activity. Further efforts are under way to determine the exact mechanism by which NDK causes toxic effects on the host cells.

ACKNOWLEDGEMENTS

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Edited by: M. Whiteley