The *Pseudomonas aeruginosa* ExoY phenotype of high-copy-number recombinants is not detectable in natural isolates

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The nucleotidyl cyclase ExoY is an effector protein of the type III secretion system of *Pseudomonas aeruginosa*. We compared the cyclic nucleotide production and lung disease phenotypes caused by the ExoY-overexpressing strain PA103ΔexoUexoT::Tc pUCPexoY, its vector control strain PA103ΔexoUexoT::Tc pUCP18, its loss-of-function control PA103ΔexoUexoT::Tc pUCPexoY K81M and natural ExoY-positive and ExoY-negative isolates in a murine acute airway infection model. Only the *P. aeruginosa* carrier of the exoY-plasmid produced high levels of cUMP and caused the most severe course of infection. The pathology ascribed to ExoY from studies using the high-copy-number plasmid on mammalian cells in vitro and in vivo was not observed with natural *P. aeruginosa* isolates. This indicates that the role of ExoY during infection with real-life *P. aeruginosa* still needs to be resolved.

1. Introduction

The type III secretion system (T3SS) of *Pseudomonas aeruginosa* enables the bacterium to inject the T3SS-associated effector proteins ExoS, ExoT, ExoU and ExoY directly into host cells via a needle-like structure [1]. In most cases, functional expression of ExoS and ExoU is mutually exclusive [2]. Both ExoS and ExoT—sharing the highest homology out of the four known T3SS enzymes—exhibit ADP-ribosyltransferase activity, interfering with manifold signalling pathways in the host cell, such as the Ras-signal transduction [2–5]. By contrast, ExoU causes direct cytotoxic effects on host cells by its phospholipase A2 activity [6]. While ExoS, ExoT and ExoU are well established virulence factors of *P. aeruginosa*, little is known about the role of ExoY during *P. aeruginosa* infection.

The effector protein ExoY was originally described as an adenylyl cyclase with structural similarities to the bacterial cyclases CyaA from *Bordetella pertussis* and oedema factor (EF) from *Bacillus anthracis* [7], having no significant impact on cytotoxicity in vitro, which led to the persisting evaluation of the exotoxin as having no clinical relevance [8,9]. Contrary to this, several recent studies have been published on a *P. aeruginosa* mutant bearing an additional plasmid coding for exoY (PA103ΔexoUexoT::Tc pUCPexoY) [7]. In these studies, a distinct phenotype of cells or animals infected with the ExoY-overexpressing mutant could be demonstrated [10–13].

ExoY synthesizes numerous cNMPs [14–16]. cUMP turned out to be the most prominent cyclic nucleotide generated in the lungs of mice infected
2. Material and methods

2.1. Cultivation of bacteria

Bacterial stocks (80% Luria Bertani (LB) broth/20% glycerol) were stored at −80°C. For experiments the recombinant *P. aeruginosa* strains PA103ΔexoUexoT::Tc pUCPexoY hereafter designated ‘ExoY’, PA103ΔexoUexoT::Tc pUCPexoY K81M and PA103ΔexoUexoT::Tc pUCP18 carrying plasmids with exoY wild-type sequence, the loss-of-function mutation K81M exoY and the empty plasmid, respectively. The latter three strains have been used in the literature to dissect the function of exoY in the absence of other T3SS effectors, but the side-by-side comparison with natural isolates has not yet been performed although this direct comparison provides a clue about the physiological relevance of phenotypes generated by a recombinant strain carrying multiple copies of exoY in *trans*.

2.2. DNA preparation

For preparation of genomic DNA, strains ExoY and ExoYK81M were washed from LB plates containing carbenicillin in a total volume of 5 ml PBS and pelleted by centrifugation; 5 ml liquid cultures of strains B420 and PT22 were pelleted as well. DNA was then prepared from bacterial cells following standard procedures which had been optimized for Gram-negative bacteria [22].

2.3. ExoY real-time PCR

Multwell PCR (StepOnePlus, Applied Biosystems) was performed with 1 ng genomic DNA per well, 50 nM primer solution (5′-GGA CGG ATT CTA TGG CAG GG-3′, 5′-CGT CGG TGT GGT GAA ACA TC-3′), 7 µl H₂O and 10 µl Power SYBR Green PCR Master Mix (Life Technologies, Germany) and normalized to the Ct-value of the *hydrogen cyanide synthase subunit* (*hcnB*) gene located adjacent to exoY in the *P. aeruginosa* genome.

2.4. Murine airway infection model

Eight- to 10-week-old female C57BL/6J mice (Janvier, Germany) were maintained in the animal facility of Hannover Medical School in microisolator cages with filter top lids at 21 ± 2°C, 50 ± 5% humidity and a 14:10 L:10 D cycle. They were supplied with autoclaved, acidulated water and fed ad libitum with autoclaved standard diet. Prior to infection mice were anaesthetized (5 mg midazolam kg⁻¹ and 100 mg ketamine kg⁻¹) intraperitoneally and to reduce anaesthesia-induced salivation each animal received atropine (dose: 1 µg per animal) subcutaneously half an hour before. Bacteria were adjusted to 10⁶ cfu and in a volume of 50 µl PBS instilled intratracheally (i.t.) to the mice lungs as described previously [23]. For the determination of the actual dosage, serial inoculates were plated on LB agar plates. Mice were sacrificed by an overdose of anaesthetic 0–72 h post-infection. Blood was taken by puncture of the...
right heart ventricle and broncho-alveolar lavage (BAL) was performed using 1 ml PBS. Individual lung lobes were weighed and used for mass spectrometric analysis of cyclic nucleotides and for histology.

2.5. Disease score, temperature, body weight and lung score

During infection mice were monitored regularly for 72 h (4, 6, 8, 10, 12, 24, 48, 72 h) by rectal temperature and body weight. The overall health was assessed by a multiparametric disease score as described before [23]. In brief, vocalization, piloerection, posture, locomotion, breathing, curiosity, nasal secretion, grooming and dehydration were recorded and dysfunctions determined by 0, 1 or 2 points. Adding these points resulted in the following score: unaffected (0–1); slightly affected (2–4); moderately affected (5–7); severely affected (8–10); moribund (greater than or equal to 11). Inflammation in infected lungs was assessed using a semi quantitative pathohistological score. Shortly, lung histological changes were scored on a scale from 0 to 2 points (no pathologic alteration = 0, mild pathologic changes = 1, severe pathologic changes = 2). Points were given separately for macroscopic evaluation of the lung tissue (visual anomalies as haemorrhage, atelectasis, 0–2), thoracic bleeding (0–1) and BALF (content of blood, 0–2) and microscopic analyses of lung tissue (oedema, apoptosis and inflammatory influx, 0–2) yielding a sum score ranging from 0 to 7.

2.6. Histology

For histology, lungs from mice sacrificed 2, 12 and 72 h after infection, were fixed with 4% formalin (v/v) and embedded in paraffin. The paraffin blocks were cut into 4 mm slices and stained with haematoxylin/eosin (Merck, Darmstadt, Germany). Microphotographs were performed using a Zeiss Axiovert 200M microscope and a Zeiss Axio Scan.X1 scanner. Exemplarily, micrographs of each group are presented in figure 2b or c.

2.7. Mass spectrometry

Tissues (50–200 mg) were transferred to 2 ml Fastprep vials containing 200 mg garnet matrix and one 1/2-inch ceramic sphere (lysing matrix A). Eight hundred microlitres of organic extraction solvent (70/30 ethanol/water [v/v]) containing 12.5 ng ml⁻¹ of the internal standard tenofovir were added and tissue was homogenized using a Fastprep-24 system (MP Biomedicals, Santa Ana, CA) at a speed of 5 m s⁻¹ for 60 s. Phosphodiesterases were inactivated by heating the homogenate for 15 min at 95°C. After centrifugation (20800g, 10 min, 4°C), 600 μl of the supernatant fluid were dried at 40°C under a gentle nitrogen stream. The residual pellet was dissolved in 150 μl water and analysed by HPLC-MS/MS as described earlier [16, 24–27]. Chromatographic data were collected and analysed with Analyst 1.5.1 software (ABSCIEX). The LLOQ for standard cAMP was 0.04 pmol per sample, for standard cGMP 0.07 pmol per sample, for standard cCMP 0.07 pmol per sample, and for standard cUMP 0.4 pmol per sample [25].
The ExoY recipients, however, continuously deteriorated during the observation period of 72 h. Lung histology revealed a similar outcome (figure 2b). Twelve hours after the instillation of bacteria inflammatory cells had emigrated into the lungs of all mice irrespective of the inoculated P. aeruginosa strain. By 72 h the number of inflammatory cells had declined in recipients of B420, PT22, ExoYK81M or DExoY bacteria, whereas cellular infiltration and inflammation had increased in mice which had received the ExoY recombinant strain. These data demonstrate that the absence or presence of a single T3SS operon did not significantly affect the course of the acute airways infection in our murine model, but that a high copy number of plasmid-borne exoY despite the absence of exoU and exoT is sufficient to induce a substantially more severe course of local and systemic infection.

3.3. Concentrations of cNMPs in lung tissue and serum of infected mice

ExoY is a promiscuous nucleotidyl cyclase that synthesizes numerous cNMPs including the previously undescribed cUMP. We measured cNMP concentrations in lung tissue and serum during the acute murine airway infection with P. aeruginosa. Fluctuating levels of cAMP were recorded in all mice demonstrating that the production of cAMP was not influenced by the absence or presence of a T3SS operon or of a functional ExoY (figure 3). Some cGMP and cCMP were detectable in lungs of ExoYK81M recipients ruling out that these cyclic nucleotides had been synthesized by ExoY. By contrast, high cUMP levels in both lungs and sera were exclusively measured in samples from mice that had been infected with the P. aeruginosa carrier of the exoY-plasmid. Thus neither the murine host nor P. aeruginosa chromosome-derived gene products but plasmid-borne ExoY had synthesized cUMP in the infected animals.

4. Discussion

The ExoY-overexpressing recombinant P. aeruginosa strains ExoY and its loss-of-function control ExoYK81M have been used as informative tools to resolve the action of the exotoxin on mammalian cells in vitro and in vivo. Thereby ExoY was identified to be a promiscuous cyclase that synthesizes...
preferentially cUMP and cGMP in vitro [14], and mainly cUMP in vivo [15]. ExoY intoxication has been shown to hinder vascular repair following infection [11], to induce intercellular gap formation and to stimulate endothelial cell tau hyperphosphorylation and insolubility [10,11,13]. Hence ExoY may drive a proteinopathy of the endothelium in the infected host [13].

The outcome of this study does not contradict these findings on the action of the exotoxin ExoY. However, our data demonstrate that the recombinant PA103 strain is strongly overexpressing ExoY thanks to the presence of multiple copies of exoY in extrachromosomal plasmids. The engineered ExoY strain caused substantial morbidity and pathology in our murine infection model, but no difference was seen between the ExoY-positive PT22, the ExoY negative B420, the ExoY knock-out ExoYK81M and the vector-negative control ΔExoY. Our findings demonstrate that the reported [10,11,13] severe infectious phenotypes are caused by multi-copy plasmid-borne exoY. Thus the role of ExoY during infection with real-life P. aeruginosa remains elusive. ExoY may indeed be an exotoxin that stimulates an infectious proteinopathy, but up to now this phenotype has not been detected by the commonly applied infection models with natural P. aeruginosa strains. However, it must be kept in mind that these infection models focus on the role of ExoY in acute infections, whereas chronic infection models may uncover some specific ExoY-associated pathology. But at present we would like to conclude that earlier statements are still valid—that wild-type concentrations of ExoY ‘have little effect on virulence and cytotoxicity’ [8]. It remains to be seen whether the F-actin mediated stimulation of ExoY activity observed in vitro [28] under certain conditions translates in vivo.

**Ethics.** All animal procedures were approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, AZ 33.14-42502-04-13/1196) and carried out according to the guidelines of the German Regulations for Animal Protection.

**Data accessibility.** All relevant data are presented in the figures in the manuscript.

**Authors’ contributions.** A.M. and C.K. designed the study, conducted experiments and drafted the manuscript. C.K. analysed the pathohistology. J.R. carried out the infection experiments and performed the real-time PCR. B.S. participated in the infection experiments, real-time PCR and sample preparation for mass spectrometry. J.K. screened P. aeruginosa strains for ExoY expression and selected the isolates used in the study. V.K. was responsible for mass spectrometry. R.S. contributed to study design and data interpretation. B.T. participated in the design of the study and in the writing of the manuscript. All authors read and approved the final manuscript.

**Competing interests.** The authors declare that they have no conflict of interest.

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