The chromosomal *pezT* gene of the Gram-positive pathogen *Streptococcus pneumoniae* encodes a protein that is homologous to the zeta toxin of the *Streptococcus pyogenes* plasmid pSM19035-encoded epsilon-zeta toxin-antitoxin system. Overexpression of *pezT* in *Escherichia coli* led to severe growth inhibition from which the bacteria recovered ~3 h after induction of expression. The toxicity of *PezT* was counteracted by *PezA*, which is encoded immediately upstream of *pezT* and shares weak sequence similarities in the C-terminal region with the epsilon antitoxin. The *pezAT* genes form a bicistronic operon that is co-transcribed from a σ70-like promoter upstream of *pezA* and is negatively autoregulated with *PezA* functioning as a transcriptional repressor and *PezT* as a co-repressor. Both *PezA* and the non-toxic *PezA*₂*PezT*₂ protein complex bind to a palindrome sequence that overlaps the promoter. This differs from the epsilon-zeta system in which epsilon functions solely as the antitoxin and transcriptional regulation is carried out by another protein designated omega. Results from site-directed mutagenesis experiments demonstrated that the toxicity of *PezT* is dependent on a highly conserved phosphoryltransferase domain sequence that overlaps the promoter. This differs from the *pezT* gene of the Gram-positive pathogen *Mycobacterium tuberculosis* (5) and *Bacillus subtilis* (6) have been demonstrated to be functional endoribonuclease and are lethal when overexpressed in *E. coli*.

Toxin-antitoxin (TA) systems were initially discovered on low copy number plasmids where they function as post-segregational killing systems that help ensure the segregational stability of plasmids. TA systems usually consist of two genes: the toxin gene encodes a stable protein whereas the antitoxin gene encodes either a labile protein or an untranslated, antisense RNA species. The toxic effect is neutralized by inhibition of toxin translation when the antitoxin is an RNA (type I), or by strong binding of the cognate antitoxin when the antitoxin is a protein (type II). When encoded on plasmids, TA systems were known as “addiction modules,” because cells which lose these plasmids would be killed, thus causing the cells to be “addicted” to the short-lived antitoxin product because its de novo synthesis is essential for cell survival (1, 2).

Over the past few years, homologs of these plasmid-borne TA systems have been identified on the chromosomes of *Escherichia coli* and various other bacteria. The discovery of the *E. coli*-encoded *mazEF* TA system led to the postulation that TA modules may function as mediators of a type of programmed cell death in bacteria, because the transcription of *mazEF* was found to be inhibited by various environmental stresses such as nutrient starvation (3). The MazE antitoxin is relatively unstable compared with the MazF toxin and any inhibition of *mazEF* transcription would “activate” MazF and lead to cell death. *E. coli* was therefore hypothesized to undergo altruistic cell death, thus helping to ensure the survival of the population during adverse conditions (3). The MazF toxin was found to exert its lethality by functioning as a sequence-specific endoribonuclease (4). Since then, several homologs of MazF from *Mycobacterium tuberculosis* (5) and *Bacillus subtilis* (6) have been demonstrated to be functional endoribonucleases and are lethal when overexpressed in *E. coli*.

Another toxin that was found to act as an endoribonuclease is the RelE toxin of the relBE TA system encoded on the *E. coli* chromosome. However, RelE was reported to cleave mRNA molecules within ribosomes but not as free transcripts like for MazF, and the RelE-mediated cleavage of mRNA is dependent on translation (7). Experiments conducted with RelE showed that while the toxin-afflicted cells cannot form colonies, they do not lyse and can be rescued from this “dormant” state by subsequent expression of the cognate antitoxin (8). TA systems were therefore proposed to be stress response elements rather than mediators of programmed cell death, as these toxins induce a reversible stasis that enable cells to survive episodes of extreme nutritional stress (2).
A survey of sequenced genomes indicated the widespread occurrence of TA systems among free living prokaryotes and these chromosomally encoded TA systems could be divided into eight families: RelBE, ParDE, HigBA, VapBC, MazEF, Phd/Doc, CcdAB, and epsilon-zeta (2, 9). To date only TA systems that belong to the MazEF and RelBE families have been extensively studied. The epsilon-zeta system was initially identified as an addiction module encoded on plasmid pSM19035 of *S. pyogenes* with epsilon functioning as the cognate antitoxin to the zeta toxin. The epsilon, zeta, and omega genes form an operon on pSM19035 with the omega gene product acting as a repressor of transcription for omega-epsilon-zeta along with other transcription units on pSM19035 (10, 11). The products of the epsilon and zeta genes were shown to form a stable epsilon2zeta2 heterotetramer (12, 13). The toxic protein zeta contains a highly conserved Walker-A motif for ATP/GTP binding, as well as a phosphotransferase active site. Epsilon neutralizes the toxicity of zeta by blocking access to the phosphotransferase active site through formation of a tight complex with zeta (12). However, the mode of action of the zeta toxin remained elusive.

A homolog of zeta was found to be encoded in the genome of the Gram-positive pathogen *S. pneumoniae* which harbors at least five other TA loci (12). Two of these pneumococcal TA loci, namely relBE-2Spn and yefM-yeoBSpn, were recently shown to be functional TA systems (14, 15). In this report, we present the crystal structure of the pneumococcal epsilon-zeta homolog, designated PezAT (for pneumococcal epsilon-zeta) and show that it is a functional TA system, with PezA as the cognate antitoxin to the PezT toxin. We also show that *pezAT* is negatively autoregulated and, unlike the omega-epsilon-zeta system, PezA functions as a transcriptional repressor with PezT as a co-repressor.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—*E. coli* DH5α was used as a host strain for most of the cloning experiments and transcriptional analyses using *lacZ* fusions. Cloning of recombinant bait and target vectors for bacterial two hybrid assays was performed in *E. coli* XL-Blue MRF' Km (Stratagene). BacterioMatch II *E. coli* validation reporter (Stratagene) was co-transformed with both bait and target pairs to enable detection of protein-protein interactions through transcriptional activation of the HIS reporter gene. *E. coli* BL21(DE3)/pLysS was used as the host strain to investigate the effect of the expression of the *pezAT* genes on cellular growth and *E. coli* BL21(DE3)/Codon Plus-RIL strain (Stratagene) was used for overexpression of the PezA and PezT proteins for purification. All cultures were grown in LB medium (Difco) at 37 °C unless otherwise stated. When necessary, the growth media was supplemented with antibiotics at the following concentrations: ampicillin (Ap), 100 μg/ml; kanamycin (Km), 50 μg/ml; chloramphenicol (Cm), 34 μg/ml for maintenance of *E. coli* BL21 strains, and 10 μg/ml for the bacterial two-hybrid assay strains.

**Construction of Plasmids for Overexpression of PezA and PezT in E. coli**—The *pezT* open reading frame was PCR-amplified from the *S. pneumoniae* genome using the primer pair PezT-F and PezT-R (for these and all other primer sequences see supplemental Table 1). The 762-bp PCR fragment obtained was initially cloned into the pGEM-T Easy PCR cloning vector (Promega). The resulting recombinant pGEM-PezT plasmid was digested to release the cloned *pezT* fragment, which was then ligated into pET11a to generate a pET11a-PezT plasmid. Similarly, a DNA fragment spanning both the *pezA* and *pezT* genes was PCR-amplified using the primers PezA-F and PezT-R, producing a single 1236-bp PCR product, which was then digested and cloned into pET11a resulting in a construct designated pET11a-PezAT. For separate expression of PezA, the open reading frame was cloned into pET28a by amplification with the primers PezA-EcoRI-F and PezA-Xhol-R and the restriction digested 477-bp fragment was ligated into pET28a resulting in a pET28a-PezA recombinant plasmid. For protein purification, both the *pezAT* and *pezA* open reading frames were each cloned into the pET28a vector such that their expression would result in an N-terminal hexahistidine fusion protein. The construct expressing the *pezAT* open reading frame, pET28a-(His)₆-PezAT, was obtained using the primers PezA-F and PezT-R for amplification, whereas the *pezA* construct, designated pET28a-(His)₆-PezA, was generated with the primers PezA-F and PezA-R for amplification. Site-directed mutagenesis was performed as described in Ref. 16.

**Cell Viability Assay**—*E. coli* BL21(DE3)/pLysS cells were individually transformed with either pET11a-PezT or pET11a-PezAT and co-transformed with pET11a-PezT together with pET28a-PezA. Overnight cultures of *E. coli* BL21(DE3)/pLysS carrying the recombinant pET11a plasmids were diluted to an A₆₅₀ of ~0.02 in fresh LB broth supplemented with Ap and Km and cells were allowed to grow at 37 °C to an A₆₅₀ of ~0.45. Cells carrying pET28a recombinant plasmids were grown in the presence of Km instead of Ap. Overexpression of the cloned genes was induced at mid-log phase (A₆₅₀ ~ 0.45) by addition of 1 mM IPTG and A₆₅₀ values were monitored at hourly intervals for up to 7 h following induction. At the same time intervals, aliquots of the cultures were taken to determine the number of viable cells. Identical overexpression experiments were also conducted with strains grown in M9 minimal medium (Fermentas).

**Overexpression and Purification of Recombinant (His)₆-PezAT and (His)₆-PezA**—*E. coli* BL21(DE3)/Codon Plus-RIL were transformed with plasmids pET28a-(His)₆-PezAT or pET28a-(His)₆-PezA. Cells were grown in LB-medium to an A₆₅₀ of 0.5 at 37 °C, cooled to 20 °C and protein overproduction was induced with 1 mM IPTG overnight. Selenomethionine labeling was performed according to Ref. 17, and induction was performed similarly to wild-type protein. For purification of the PezAT protein complex, cells were harvested by centrifugation and the cell pellet was resuspended in buffer A (see supplemental Table S2 for buffer compositions). Cell walls were broken by sonication, and the slurry was cleared by centrifugation. Affinity chromatography was performed using a Ni-NTA material (Qiagen) equilibrated with buffer A. Unbound sample was removed by washing with buffer A. The protein-complex was eluted with buffer A with additional 250 mM imidazole. Fractions containing the PezAT protein complex were pooled and the N-terminal hexahistidine-tag of PezA was removed by
thrombin (GE Healthcare) cleavage in a dialysis against cleavage buffer B at 4 °C for 12 h. The protein-complex was further purified over a MonoQ column (GE Healthcare). Briefly, the dialysed PezAT protein complex was diluted with buffer C and loaded onto the column. The PezAT proteins were eluted in a gradient of up to 600 mM NaCl and were subjected to a final clean-up by size exclusion chromatography using Superose 6 (GE Healthcare) equilibrated with buffer D. Selenomethionine-labeled PezAT proteins were purified similarly to the wild type protein with the addition of 5 mM DTE throughout all purification steps.

For purification of PezA, harvested cells were resuspended in buffer I, and the cells were lysed by sonication. The cell debris was cleared by ultracentrifugation and the crude lysate was subsequently subjected to ammonium sulfate fractionation to a final concentration of 40% saturation. After centrifugation, the pellet was dissolved in and dialyzed against buffer J. The proteins were further applied onto a HisTrap HP affinity column (GE Healthcare) equilibrated with buffer J. Following extensive washing with buffer J, the bound proteins were eluted from the column using buffer J containing 500 mM imidazole.

For purification of the mutated variant PezT(K45A), harvested cells were resuspended in buffer E. After cell lysis by sonication, the cleared supernatant was loaded over a HisTrap FF column (GE Healthcare) equilibrated with buffer E. Non-specifically bound proteins were removed in a high salt wash with buffer E containing additional 500 mM NaCl, and bound proteins were eluted in a gradient to 500 mM imidazole. The pooled protein fractions were diluted with buffer F prior to loading on a heparin column (GE Healthcare) equilibrated with buffer F. Following extensive washing with buffer J, the bound proteins were eluted from the column using buffer J containing 500 mM imidazole.

For crystallization experiments, the protein was concentrated to 17 mg/ml. Protein concentrations were determined by the Bradford assay (Bio-Rad). Protein purity, complete cleavage of the hexahistidin tag and incorporation of selenomethionine were monitored by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF MS).

**Mass Spectrometry**—MALDI-TOF mass spectrometry analysis was performed in the linear, positive ion mode with blanking (<700 m/z) and pulsed (time-delayed) extraction using a Shimadzu Biotech Axima TOF2 instrument (Shimadzu Biotech Germany). All reagents and protein standards were purchased from Sigma-Aldrich. Sinapinic acid (10 mg/ml in 50% acetonitrile, 50% of 0.1% trifluoroacetic acid (all % v/v)) was used as a matrix. Sample positions on the steel 384-position sample plate were washed once with matrix solution. A 1-μl drop of the protein preparation or 1 μl of water into which the crystal was transferred with the cryoprotectant was allowed to dry onto the sample plate at room temperature and the preparation was then washed with a small drop of 0.1% trifluoroacetic acid applied for ~10 s. Immediately after removal of the trifluoroacetic acid, 1 μl of matrix solution was added to the sample and it was allowed to dry at room temperature. The standard proteins (about 1 pmol of insulin, ubiquitin, cytochrome c, myoglobin, chymotrypsinogen A, aldolase, bovine serum albumin, catalase, myoglobin, and β-galactosidase) were spotted onto the washed plate and an equal volume (usually 1 μl) of matrix was immediately added to the protein drop. Each protein standard was analyzed separately and a combined calibration of the near external standards was employed to determine the mass/charge (m/z) values.

**Isothermal Titration Calorimetry**—ITC was performed using a VP-ITC microcalorimeter (MicroCal Inc.). Data were analyzed using the Origin-based software provided by the manufacturer. A buffer exchange to buffer D was performed by size exclusion chromatography and protein concentrations were determined according to their absorbance at 280 nm. Titrations were carried out at 15 °C and, in a typical experiment, 3 μM PezT(K45A) were loaded into the sample cell and titrated against 30 μM PezA in the injection syringe.

**Protein Crystallization and Data Collection**—Crystals of the PezAT protein complex were obtained from drops made of 1.5 μl of protein solution and 1.5 μl of precipitant solution (12.5 to 15% (v/v) isopropyl alcohol, 100 mM MES-NaOH, pH 6.0) in a hanging-drop vapor diffusion setup at 293 K. Crystal size was optimized by adding 1 to 6% (v/v) of a 30% (v/v) dioxane stock solution to the mixed crystallization drop. Single individual crystals grew to a maximum size of about 300 × 100 × 50 μm³ within 2 weeks. Before flash-cooling in liquid nitrogen, crystals were harvested into precipitant solution with additional 10% (v/v) glycerol, and the glycerol concentration was increased stepwise to 30% (v/v) for cryo protection. Native and anomalous diffraction data were collected at beamline PXII at the Swiss Light Source with the crystals kept at 90 K. The datasets were processed and scaled using XDS (18). The selenium sites were located with the program SHELXD (19). Initial phase improvement and density modification was performed with SHELXE (20). Model building was performed with the program "O" (21) and refined using CNS (22) in the beginning and REFMAC (23) in the later stage of refinement. The overall quality of the structure was evaluated by PROCHECK (24).

**In Vivo Detection of Protein-Protein Interaction Using a Bacterial Two-hybrid System**—The BacterioMatch II Two-Hybrid System Vector Kit (Stratagene) was employed according to the manufacturer’s instructions to investigate in vivo interactions between PezA and PezT. Both pezA and pezT were separately cloned as fusion proteins with the αC1 repres sor into pBT as bait constructs and in frame with the RNAAPα reading frame into pTRG as target vectors. In the case of pezA, DNA fragments were amplified using the primer pair BPezA-F and BPezA-R and cloned into the pBT bait vector for the bait construct pBT-PezA. The recombinant target of pezA was generated by PCR using primer pair TPezA-F and TPezA-R and cloned into pTRG for the target construct pTRG-PezA. For generation of bait and target constructs of pezT, an intermediate cloning step into the pGEM-T Easy PCR cloning vector (Promega) after amplification with the primer pairs PezT1-F and PezT1-R was introduced. Following restriction digest, the isolated pezT DNA fragment was ligated into pBT for the bait construct pBT-PezT and into pTRG for the target construct pTRG-PezT. The E. coli reporter strain (Stratagene) was co-transformed with each of the recombinant bait and target pairs (i.e. pBT-PezA and pTRG-PezT pair, or pBT-PezT and pTRG-PezA pair). Pos-
itive interaction between the lacI-fusion protein produced by the recombinant pBT and the RNApol–fusion protein produced by the recombinant pTRG was monitored by growth of the reporter strain co-transformants on the selective medium containing 5 mm 3-amino-1,2,4-triazole (3-AT).

RNA Preparation and Reverse Transcriptase-PCR (RT-PCR)—Total RNA samples were prepared from E. coli cultures using an RNeasy Mini kit (Qiagen) according to the manufacturer’s protocol. The total RNA concentrations were determined by UV spectrophotometry, and the sample integrity was checked by agarose gel electrophoresis. RT-PCR was carried out using 400 ng of total RNA with the Access RT-PCR System (Promega) according to the manufacturer’s procedure. The gene specific primers 1050-F, 1050-R, 1051-F, and 1051-R were used in the RT-PCR analysis.

Construction of lacZ Transcriptional Fusions and β-Galactosidase Assays to Detect Promoter Activity—Four constructs spanning different regions of the pezAT operon were amplified and subsequently ligated into the vector pQF52 (25) to obtain different lacZ transcriptional fusions for the detection of promoter activity. First, the putative promoter region upstream from pezA was amplified using the primer pair upPezA-F and upPeza-R yielding a 209-bp fragment, which was cloned into pQF52 resulting in the pQF-PezAup construct. Second, the promoter region together with pezA was amplified (using the upPeza-F and lacZ_PezA-R primers) as a 712-bp fragment, which was then cloned to generate pQF-PezA. Third, the entire pezAT operon (1388-bp DNA fragment) along with its promoter region was obtained with the primer pair upPezA-F and lacZ_PezT-R for the recombinant pQF-PezAT. Finally, pQFPezATup was constructed by ligation of pQF52 with a 233-bp fragment that contained a truncated pezA reading frame that was amplified using the primer pair PezA283-F and PezA-R. E. coli DH5α was transformed with the recombinant pQF52 plasmids and transformants were assayed for β-galactosidase activity (when A₆₆₀ reached ~0.5) according to the method of Miller (26), using SDS and chloroform to permeabilize the cells.

Determination of lacZ Transcript Levels using Quantitative Real-time RT-PCR—The LightCycler RNA master SYBR Green I kit (Roche Applied Science) was used to carry out quantitative real-time RT-PCR on the LightCycler 2.0 (Roche Applied Science) for the detection of lacZ transcript levels using the primer pair lacZ-F and lacZ-R. Total RNA from E. coli DH5α harboring the various pQF-derived recombinants were extracted using the RNeasy mini kit. Reverse transcription was carried out at 61 °C for 20 min, and the cDNA/RNA hybrid was then denatured at 95 °C for 30 s. After that, 45 cycles of real-time PCR amplification were carried out using the following parameters: denaturation for 1 s at 95 °C, annealing for 5 s at 5 °C below the predicted melting temperature of the primer pair, and elongation for 13 s at 72 °C. The E. coli-encoded 16S rRNA gene was used as a housekeeping control (using the primer pair 16S-F and 16S-R). The relative expression ratio of the target gene, lacZ, was calculated based on its real-time PCR efficiencies (E) and the crossing point difference (ΔCP) for an unknown sample versus a control using the mathematical model published by Pfaffl (27). For each gene (i.e. lacZ or 16S rRNA), cDNA dilution curves were generated and used to calculate the individual real-time PCR efficiencies with the values for the slopes obtained using the LightCycler 4.0 software (Roche Applied Science). For each sample, at least three different real-time RT PCR reactions were carried out from which the mean CP value was obtained utilizing the LightCycler 4.0 software (Roche Applied Science).

Determination of the Transcriptional Start Site using 5’-RACE—The transcriptional start site of the pezAT operon was determined using 5’-RACE (Version 2.0; Invitrogen), following the manufacturer’s protocol. Total RNA was extracted from E. coli DH5α harboring the pQF-PezAT recombinant plasmid and was subjected to first strand cDNA synthesis using 20 ng of GSP1 primer, which was designed to anneal 399-bp downstream of the pezA start codon. A homopolymeric tail was then added to the 3’-end of the synthesized cDNA using terminal deoxynucleotransferase and dCTP. The dC-tailed cDNA was then PCR-amplified using the provided anchor primer, which was complementary to the homopolymeric tail, and a nested primer, GSP2, located 121-bp downstream of the pezA start codon. The amplified product was purified and sequenced to determine the transcriptional start site, which would be the nucleotide that is immediately after the poly(G) tail.

Electrophoretic Mobility Shift Assays—A 203-bp DNA fragment containing palindromic sequence PS, which is the putative regulatory protein binding site, was PCR-amplified using the primer pair PS-F and PS-R, purified and labeled with biotin using the Biotin 3’-End DNA Labeling kit (Pierce). Subsequent electrophoretic mobility shift assays were carried out using the LightShift chemiluminescent EMSA kit (Pierce). Biotinylated DNA (20 fmol) was incubated at room temperature for 20 min with 50 ng/μl polyoxyninosinic-polycyctydlylic acid [poly(dI-dC)] and different amounts of purified protein (either PezA or the PezAT protein complex) in binding buffer K. Nucleoprotein complexes were separated from free DNA by electrophoresis on a 6% polyacrylamide gel in 0.5× Tris borate/EDTA buffer and subsequently transferred onto a nylon membrane. The biotin end-labeled DNA was detected using a streptavidin-horseradish peroxidase conjugate and the supplied chemiluminescent substrate.

RESULTS

Sequence Analysis—A homolog of the S. pyogenes plasmid pSM19035-encoded zeta toxin was found in the genome sequence of S. pneumoniae TIGR4. The PezT protein encoded by the sp1051 reading frame shares 42% sequence identity with zeta (Fig. 1A) suggesting that both proteins have similar functions in different Streptococcal species. However, a BLAST search for the corresponding antitoxin homolog to the epsilon protein in the genome sequence of S. pneumoniae did not yield any positive results. As TA systems are usually organized as operons, the reading frames that flank sp1051 were further analyzed. Interestingly, pairwise amino acid sequence alignment of the upstream open reading frame sp1050 gene product PezA with epsilon indicated that the C-terminal residues 70–158 of PezA share 21% sequence identity with epsilon (Fig. 1A). Apparently arranged in a bicistronic operon, the sp1050 (pezA) reading frame overlaps with sp1051 (pezT) by one nucleotide, where the last A nucleotide of the TAA stop codon of pezA is
also the start of the ATG initiation codon of \textit{pezT}. This arrangement is similar to that found in the other two \textit{S. pneumoniae} genomes that have been sequenced, strains R6 (designated spr0951–spr0952; accession no. NC_003098) and D39 (designated SPD_0930-SPD_0931; accession no. CP000410). Furthermore, we found that the N-terminal 70–80 amino acids of spr0951--spr0952; accession no. NC_003098) and D39 (designated SPD_0930-SPD_0931; accession no. CP000410). Furthermore, we found that the N-terminal 70–80 amino acids of...
PezA share 30 to 40% sequence identity with transcriptional regulators of the Xre and Cro/Cl families. Thus pezAT was investigated to determine if the two genes encode for a functional toxin-antitoxin pair that is autoregulated by PezA, which also functions as the cognate antitoxin to the PezT toxin.

Overexpression of pezAT in E. coli—To determine if pezAT is indeed a functional TA system and whether pezT encodes for the toxic protein, the pezAT genes were cloned together as well as separately into the pET11a expression vector. The pezAT genes were obtained by PCR amplification using genomic DNA from clinical isolates of S. pneumoniae as template. The recombinant plasmids were sequenced on both strands for verification. We found that the cloned pezAT sequences obtained from six S. pneumoniae clinical isolates differed by between 4–6% when compared with the data base sequence for the TIGR4 strain. Although most of these sequence differences led to silent mutations (no frameshift mutations were identified), the translated PezA and PezT proteins do differ by up to 3% with the TIGR4 strain (Fig. 1A). A similar degree of variation was found when the pezT sequences from the R6 and D351 strains in the data base were compared with the TIGR4 strain, thus indicating a natural degree of inherent sequence variability within this locus. The pezAT fragment showing the least variation from the TIGR4 sequence was chosen for this study. Here, the PezA amino acid sequence is identical with the TIGR4 sequence whereas the PezT sequence showed five differences: K26R, R109G, V121I, K146R, and A220E. Two of these differences, R109G and V121I, were also found in the PezT sequences of the R6 and D351 strains. Except for R109G, all non-homologous sequence variations observed within the PezT family align with non-conserved amino acids between PezT and zeta (Fig. 1A).

Interestingly, all isolated variants had a glycine replacement for residue Arg^{109}. Whereas cloning of both pezA and pezT on a single DNA fragment proceeded without any problems, cloning of the toxin-encoding pezT was difficult and was successful only after several attempts. Similar problems had also been reported for the epsilon/zeta locus (28).

In cell viability assays, overexpression of PezT resulted in growth inhibition with a reduction of $\sim 10^5$ in cell viability for the first hour post-induction. The reduction in cell growth was even more drastic for the subsequent 2 h where the viable counts dropped by $\sim 10^8$ compared with the uninduced state (Fig. 2). However, restoration of cell growth was observed $\sim 3$-h post-induction with a gradual increase in both the A_{600} value as well as viable cell counts (Fig. 2). Following overnight growth, there were no discernable differences in both the A_{600} as well as the viable cell counts of the E. coli BL21 cells overexpressing PezT when compared with its uninduced counterpart and the negative control (data not shown). This growth profile is remarkably similar to what has been reported for the overexpression of zeta in E. coli (28). The similar experiment was repeated by growing the cells in M9 minimal medium instead of LB broth which revealed an almost identical growth profile (data not shown). Sequencing of plasmid DNA isolated from cells that have recovered from stasis did not reveal any mutations in the pezT gene nor in the T7 promoter region of the pET11a expression vector.

This inhibition in cell growth was not observed when pezA was co-expressed along with pezT in E. coli BL21 harboring the pET11a-pezAT construct. To investigate if neutralization of PezT toxicity by PezA could also occur in trans, a pET28a-PezA plasmid was co-transformed with pET11a-PezT into E. coli BL21(DE3)/pLysS and the transformants obtained were subjected to IPTG induction for protein expression. Cellular growth was not inhibited, showing that PezA was able to neutralize PezT toxicity even when the pezA reading frame was provided in trans. Taken together, these results indicate that PezT functions as a toxin that inhibits the growth of E. coli cells for the first 3 h of overexpression and this toxicity is abolished by co-expression of PezA, which apparently functions as its cognate antitoxin.

PezA Interacts with PezT and Forms a Tight Complex—The pET28a-pezAT recombinant plasmid was constructed such that the PezA protein is expressed as a hexahistidine fusion protein from the T7 promoter of the pET28a vector. In pull-down experiments, PezT was successfully co-purified with (His)_6-PezA immobilized on Ni-NTA agarose beads. Following elution, two distinct protein bands were observed on SDS-PAGE corresponding to the expected mobilities of the (His)_6-PezA fusion protein (19 kDa) and PezT (29 kDa) indicating that under native conditions, both PezA and PezT were co-purified (Fig. 3A). Mass spectrometry demonstrated that both PezA and PezT had the expected mass.

To characterize the binding properties of PezA and PezT in detail, ITC experiments were performed. The proteins bind to each other with an apparent $K_D$ below 1 nM (Fig. 3B). This low $K_D$ is at the borderline of measurable affinities by ITC, because the signal to noise ratio decreases at the required low concentrations. When PezA was titrated with PezT, the signal slowly
The PezAT Toxin-Antitoxin System of Streptococcus pneumoniae

In vivo interactions between the PezA and PezT proteins were investigated using a bacterial two-hybrid system (see “Experimental Procedures”) where growth of the E. coli indicator strain on the 5 mM 3-AT selective medium was observed for strains that were co-transformed with pBT-pezA and pTRG-pezT (data not shown). Interestingly, when the interaction pair was switched, i.e. using pBT-pezT and pTRG-pezA, no growth of the E. coli indicator strain was detected on the selective medium.

Transcriptional Analyses of pezAT—Analyses of sequences upstream of pezA indicated the presence of a characteristic −10 promoter sequence (TATAAT) 33 nucleotides upstream of the pezA start codon as well as an imperfect −35 sequence (TTGTGT) that is separated by 18 nucleotides from the −10 hexamer (Fig. 4A). A 56-bp imperfect palindrome sequence, designated PS, overlaps both the −10 and −35 region and is centered 59 nucleotides upstream of pezA. A putative ribosome-binding site (AGGAG) could also be found 6 nucleotides downstream of the ATG start codon (Fig. 4A).

To obtain information on the activity of the putative promoter designated P_{pezAT} transcriptional fusions with a promoterless lacZ gene as a reporter in the E. coli plasmid pQF52 were carried out. The functionality of P_{pezAT} was validated when E. coli DH5α cells harboring pQF-pezAup (191 bp of the pezA upstream region cloned upstream of lacZ in pQF52) showed mean β-galactosidase activity of 130 (±25) Miller units compared with a value of less than 5 for cells harboring the pQF52 vector alone (Fig. 4D). No β-galactosidase activity could be detected in cells harboring pQF-pezTup (which contained sequences in the 193-bp region upstream of the pezT start codon) indicating no detectable promoter activity within the pezA reading frame immediately upstream of pezT.

The pezA and pezT reading frames overlap by 1 nucleotide and would appear to constitute a bicistronic operon, as in the case of other TA systems (1, 2). Total RNA was isolated from E. coli DH5α harboring the pQF-PezAT recombinant constructs, which contained the entire pezAT reading frames as well as 191 bp of sequences upstream of pezA encompassing the P_{pezAT} promoter. Reverse transcriptase was used to synthesize cDNAs that were complementary to pezAT mRNA and the resulting cDNAs were PCR-amplified using two oligonucleotide primers (1050-F and 1051-R) that would anneal to the reading frames as controls (Fig. 4C). No specific PCR products were evident in the negative controls without a reverse transcriptase extension step but still applying the same RNA samples. Possible DNA contamination can be excluded since no PCR products were evident in the negative controls without a reverse transcriptase extension step but still applying the same RNA samples.

Total RNA isolated from E. coli harboring pQF-PezAT was subjected to 5'-RACE to determine the transcriptional start site. Sequencing of the 5'-RACE products obtained from three different preparations of RNA showed that the pezAT transcript initiated at the A residue 21 bp upstream of the pezA start codon (designated +1 in Fig. 4A). Taken together, these results decreased to background level, possibly due to disassembly of PezA oligomers before PezA is able to bind to PezT. This finding is supported by size exclusion chromatography experiments, where PezA has a shorter retention time than expected, suggesting that PezA alone tends to form higher oligomeric aggregates. The obtained stoichiometric relation for the complex is around 1 suggesting that in vitro both proteins form oligomers with a stoichiometry of PezA_{n}PezT_{n}. 

FIGURE 3. A, pull-down experiment of (His)_6-PezA and PezT. Chromatogram showing elution profile of proteins from the nickel affinity column on which crude extract of E. coli BL21 Codon Plus RIL strain harboring pET28-pezAT was applied. SDS-PAGE analysis of the eluted fractions is shown in the inset. B, ITC experiment with PezA and PezT(K45A). Top, thermogram resulting from a titration of 3 μM PezT in the sample cell with 30 μM PezA in the injection syringe. Bottom, best fit (solid line) to the experimental data (filled squares). Given results were obtained from an analysis of the data assuming a single binding site model.
show that pezAT is a bicistronic operon that is transcribed from a σ70-like promoter, P_{pezAT}, the Pribnow box starting 33 bp upstream of the pezA start codon.

The PezA Antitoxin Represses Transcription from P_{pezAT} and the PezT Toxin Acts as a Co-repressor—When comparing β-galactosidase activities of E. coli DH5α harboring recombinant plasmid pQF-pezAT. Subsequent PCR reactions utilized oligonucleotide pairs as indicated by the horizontal bar; lane 4 contains the negative control, i.e., PCR reaction similar to lane 3 but without the reverse transcriptase reaction. Lane M, DNA molecular weight standard with sizes as indicated in bp. D, results of the lacZ transcriptional fusions. Schematic diagram of the various pezAT-lacZ transcriptional fusion constructs in pQF52 is shown in the table with the organization of the pezAT genes, its P_{pezAT} promoter and the palindrome sequence PS shown at the top of the table. DNA fragments containing combinations of the pezAT locus and its upstream region were cloned upstream of the promoterless lacZ (depicted as a gray box) in plasmid pQF52 resulting in various pQF52-derived recombinant constructs as depicted. Complete open reading frames are indicated as block arrows. The β-galactosidase activity levels presented are the average results of at least six independent experiments and are also presented as relative levels (in %). The relative ratios of lacZ transcript levels to 16 S rRNA as determined by real-time RT-PCR are also presented.
resulted in a 29% decrease in lacZ transcript levels compared with the levels in pQF-petzAup and this decreased further by nearly 74% in the presence of both the pezAT reading frames (Fig. 4D). Thus, the PezA antitoxin functions as a transcriptional repressor with the PezT toxin acting as a co-repressor and both proteins are therefore involved in the autoregulation of the pezAT operon, like in most other TA systems (1, 2).

To investigate whether the palindromic sequence PS upstream of pezAT is indeed the DNA binding site for the PezA and PezT proteins, electrophoretic mobility shift assays (EMSA) were performed. PezA and PezT proteins were expressed and purified as a recombinant N-terminal (His)_6-PezA fusion and a (His)_6-PezAT protein complex from E. coli as described under “Experimental Procedures.” The purified PezA protein and the PezAT protein complex were separately incubated with a biotin-labeled 203 bp DNA fragment encompassing PS. Binding reactions were carried out in the presence of 50 ng of poly(dI-dC) as a nonspecific competitor DNA. Results indicated that the majority of labeled DNA was retarded when 750 ng of PezA was included in the binding reaction (Fig. 5A). Essentially similar results were obtained with the PezAT complex where the majority of the retardation occurred with 750 ng of the protein complex but initial retardation was detected already at lower PezAT concentrations than with PezA alone (Fig. 5B).

The PezA-PezT₂ Architecture—To understand the PezAT system in detail, we solved the crystal structure of the PezAT protein complex at a resolution of 3.2 Å by the single anomalous diffraction (SAD) method. Details describing x-ray diffraction data quality and refinement statistics are given in Table 1, and the Ramachandran statistics are given in Table 2.

The apparent biological unit of the PezAT TA system is a PezA₂PezT₂ heterotetramer, which has a dumbbell-shaped arrangement with an internal 2-fold symmetry (Fig. 1B). The central core is formed by a stack of two PezA antitoxin polypeptide chains that are sandwiched between two PezT toxin molecules whose dyad axis lies parallel to the PezA homodimer interface. Overall, this molecular arrangement is very similar to the architecture observed in the crystal structure of the epsilon-zeta system (12). Despite the significant sequence homology between the two different antitoxin and toxin polypeptide chains, conserved residues on the surface of the PezA₂PezT₂ heterotetramer map only to one region (Fig. 1C), which was recently proposed to be the substrate binding side of the putative phosphoryltransferase zeta (12). Further, larger conserved patches that would suggest common modes of interaction of these two different TA systems could not be identified on the surface of the antitoxin-toxin protein complex. The majority of conserved residues is either shielded within the protein complex or is important for establishing a similar fold.

Two heterotetrameric protein complex molecules were located within one asymmetric unit in the crystal. Although the individual PezA and PezT polypeptide chains have a very similar geometry and their r.m.s.d. on Ca positions is at most 0.924 Å for all toxin and 1.225 Å for the antitoxin polypeptide chains, several regions within the polypeptide chain show distinct differences. Consequently, a non-crystallographic restraint could not be used during refinement, because such restraint caused the R-factors to increase. These differences between individual PezT polypeptide chains can be attributed to crystal contacts to neighboring molecules and probably do not reflect biological relevant conformational states of the proteins. Furthermore, some regions within the polypeptide chain of PezT did not show up in the electron density and the polypeptide chain could not be modeled for the corresponding residues (see Table 2). The major portion of the polypeptide chains that lacks electron density interpretable at atomic resolution resides within the helix-turn-helix (HTH) motif within the N-terminal region of the PezA polypeptide chain. Although we found residual elec-
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TABLE 1
X-ray diffraction data and refinement statistics

|                     | Selenomethionyl | Native |
|---------------------|-----------------|--------|
| Data collection     |                 |        |
| Space group         | P2, 2, 2        | P2, 2, 2 |
| Unit cell (a,b,c (Å)) | 81.3, 103.8, 255.0 | 80.5, 102.9, 254.4 |
| Wavelength (Å)      | 0.97908         | 1.007466 |
| Resolution (Å)      | 20-3.8 (3.9-3.8) | 10-3.2 (3.3-3.2) |
| Unique reflections   | 40,716 (3075)   | 34,352 (4708) |
| Completeness (%)    | 99.1 (99.4)     | 95.3 (85.5) |
| <(I/σ(I))>a        | 19.6 (8.1)      | 20.8 (3.1) |
| Rmerge,ab          | 6.7 (22.1)      | 6.0 (38.7) |
| Redundancy         | 6.7             | 5.7     |

| Refinement         |                  |        |
|-------------------|------------------|--------|
| Non-hydrogen atoms | 11093            |        |
| Rmerge,ab         | 21.4 (27.7)      |        |
| Rfree             | 27.7 (34.7)      |        |
| Overall B factor  | 91.27            |        |

| r.m.s.d. from ideal geometry | Bond length (Å) | 0.008 |
|------------------------------|-----------------|-------|
|                              | Bond angles (°)  | 1.059 |

| VALUES IN PARENTHESES REFER TO THE HIGHEST RESOLUTION SHELL. |

| Ramachandran statistics | Most favored region | Generously allowed region | Disallowed region |
|-------------------------|---------------------|--------------------------|-------------------|
|                         | 91.0%               | 9.0%                     | 0.0%              |

| Because of weakly defined electron density, this region was modeled as a polyglycine chain. |

The PezA Three Helix Bundle—On a primary structural level, the major difference between the PezAT and the epsilon/zeta TA systems is found between the two different antitoxin polypeptide chains. Interestingly, the C-terminal region of the PezA polypeptide chain folds into a three helix bundle similar to that of epsilon. Strikingly, residues that form the hydrophobic core of the three helix bundle are conserved, whereas residues involved in PezA homodimer formation are only moderately conserved (Fig. 1A). Surface-exposed residues of PezA are much less conserved (Fig. 1D). The lack of conservation of the antitoxin at the grip of the dumbbell-shaped molecule suggests that this region might not be important for PezA functionality.

Inactivation of PezT by PezA—In the inactive PezA PezT heterotetrameric protein complex, the nucleotide binding site of PezT is covered by two helices of PezA (helix a1 and a2, Fig. 1). Strikingly, three evolutionarily invariant residues of the antitoxin PezA (Tyr73, Leu77, and Glu80, see supplemental Fig. S1) protrude with their side chains into the putative nucleotide ATP/GTP binding proteins such as a Walker A motif or P-loop. In order to verify whether PezT and zeta indeed have the same toxic activity, we mutated residues that were previously shown to be involved in zeta toxicity (12) at equivalent positions in PezT and tested these constructs in cell viability assays. For instance, individual mutations of K45A, R157A, or R170A (Fig. 6) abolished PezT lethality. All three residues are known from related structures to be involved in ATP/GTP binding and hydrolysis (12, 29). Furthermore, an aspartate residue was shown in the latter report to deprotonate the substrate before phosphoryl transfer and mutation of the equivalent residue D66T indeed resulted in a non-toxic variant of PezT. Similar observation of the loss of toxicity was also observed in the zeta toxin (12). Consequently, both toxic proteins seem to use a similar mechanism of ATP/GTP binding, hydrolysis, and phosphoryl transfer.

The effects of the D66T mutation in PezT and the corresponding residue in zeta corroborate that a phosphoryl transfer reaction from ATP/GTP to an unknown substrate is much more likely responsible for toxicity than a simple nucleoside triphosphate hydrolysis. However, based on the epsilon-zeta structure alone, neither the substrate binding site could be experimentally verified nor could any conclusion be derived concerning the true chemical nature of the toxin substrate.

Using a combination of structural data analysis with bioinformatics tools, we can now identify residues that are located adjacent to the ATP/GTP binding site and structurally conserved between PezT and zeta (Fig. 6). These residues cluster in a groove that is formed by helix a5 and helix a6. To show that this region is indeed involved in substrate binding, we mutated two conserved threonine residues, T117V and T120V. These two threonine residues project into the groove and are in position to form hydrogen bonds with the substrate. Both mutations impaired PezT toxicity. Whereas T117V resulted in a non-toxic variant that showed a normal growth profile when expressed in E. coli BL21, the T120V mutation led to a delay in the growth inhibition, which occurred ~1 h following IPTG induction for overexpression (Fig. 6B). The duration in which cellular growth was inhibited in the T120V mutant was similar to that of the wild-type PezT, i.e. about 3 h.

The PezT Toxin Structure—PezT adopts the fold of NMP-kinase and the chloramphenicol phosphotransferase as had been reported for the zeta toxin (12) (Fig. 1B). Furthermore, PezT also contains sequence motifs that are characteristic for
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binding site of PezT and thereby prevent nucleotide binding in a manner similar to the epsilon/zeta system. 

The Helix-Turn-Helix Motif in PezA—Although we do not have an atomic model for the HTH-motif in the N terminus of PezA, the presented structure allows us to derive a hypothesis about the orientation of this domain. The HTH motif identified in the electron density is located in proximity of the dyad axis of the PezA$_2$PezT$_2$ heterotetramer. In the absence of operator

DNA, the HTH domains apparently do not form strong interactions with each other or PezT and are therefore flexible. Assuming that the overall architecture of the PezA$_2$PezT$_2$ complex remains preserved upon binding to the operator site, the two HTH domains must obey the overall 2-fold symmetry of the complex and should, in this fashion, bind to a palindromic sequence such as found for the P$_{pezAT}$ promoter.

DISCUSSION

In this study, we showed that the chromosomally encoded pezAT bicistronic operon of the human pathogen S. pneumoniae is a functional TA locus with pezA encoding the cognate antitoxin to the toxic product of pezT. Like most TA systems, the pezAT locus is autoregulated with the PezA antitoxin functioning as a transcriptional repressor by binding to a palindromic sequence that overlaps the P$_{pezAT}$ promoter and the PezT toxin acts as a co-repressor. Our in vivo and in vitro data were further corroborated by the crystal structure, where the N-terminal domain of PezA adopts the fold of a DNA binding domain with a HTH motif belonging to the Xre and Cro/CI family. Thereby, the PezAT system differs significantly from the omega-epsilon-zeta locus of the S. pyogenes plasmid pSM19035, where omega, as a third component, is the transcriptional regulator for the epsilon-zeta bicistronic operon. Epsilon functions solely as the antitoxin for zeta (11, 28, 30) and omega has been shown to bind to operator DNA via a ribbon-helix-helix motif that belongs to the MetJ/Arc superfamily of transcriptional repressors (31, 32). Apparently, the regulatory domains for transcription of the PezAT system and the omega-epsilon-zeta systems are evolutionarily not related and both systems have different transcriptional regulatory mechanisms. Similar to most TA systems, epsilon was shown to have a shorter half-life than zeta (13). However, the regulatory mechanism via which omega senses a decreasing concentration of epsilon for proper functioning of the TA system remains elusive. In case of the PezAT system, this regulation becomes immediately evident, because the transcriptional repressor and the toxin-neutralizing activity can be found within a single polypeptide chain in PezA. A decreasing amount of antitoxin would be compensated by its transcriptional repressor function and thereby PezA ensures that the toxin PezT does not become free and active.

The three-dimensional structure of the PezT toxin is very similar to zeta and mutations in equivalent residues of PezT

FIGURE 6. A, surface representation of PezT alone, colored according to Fig. 1A. Residues that are identical with zeta and showed to impair PezT toxicity are colored in blue. An ATP molecule colored in magenta was modeled according to the position found in the chloramphenicol-phosphotransferase (29). B, effect of PezT mutations on the growth of E. coli BL21(D3)/plyS carrying recombinant pET11a plasmids as determined by A$_{600}$ and log CFU. Growth profile of wild-type PezT is shown as (X) for A$_{600}$ values (top) and black bars in log CFU (bottom). Growth profiles of the following mutants following IPTG induction are shown: T117V (□, green bars), T120V (■, blue bars), and K45A (○, yellow bars). The R157A, R170A, and D66T mutations yielded nontoxic growth profiles similar to those of K45A and T117V and are not presented in the figure.
which have previously been shown to be involved in zeta toxicity also abolished PezT activity. It is therefore very likely that PezT and zeta have similar mechanisms for toxicity. Apparently, both use ATP/GTP for phosphoryltransfer to an unknown substrate. However, we could now clearly demonstrate by site-directed mutagenesis that a recently proposed substrate binding site of zeta (12) is indeed involved in toxicity. Mutation of two threonine residues that are located distal from the nucleotide binding site to valine abolished toxicity. A common mechanism is further supported by the growth profile of *E. coli* cells overexpressing PezT. Inhibition of growth was only temporary and restoration of growth occurred about 3 h after induction of overexpression, similar to the phenomenon observed in cells that express zeta. Microscopic examination of cultures during this growth inhibition period revealed massive filament formation, but no induction of the SOS system was detected (28). Preliminary examination of PezT-inhibited cultures also indicated similar morphological traits (data not shown). Recent studies showed that induction of zeta expression in *E. coli* cultures also indicated similar morphological traits (data not shown). Preliminary examination of PezT-inhibited filament formation, but no induction of the SOS system was observed in cells that express zeta. Microscopic examination of induction of overexpression, similar to the phenomenon observed in cells that express zeta. Microscopic examination of cultures during this growth inhibition period revealed massive filament formation, but no induction of the SOS system was detected (28). Preliminary examination of PezT-inhibited cultures also indicated similar morphological traits (data not shown). Recent studies showed that induction of zeta expression in *E. coli* CC118 cells led to the inhibition of DNA, RNA and protein synthesis. However, it was hypothesized that zeta exerts a pleiotropic effect on the physiological state of the cells (33). The target and mechanism of PezT/zeta lethality remains to be elucidated and is the subject of our ongoing investigations.

Although TA systems have been found to be widely distributed especially among free-living prokaryotes (9), the targets of many toxins have yet to be identified. The toxins of three well-studied chromosomal TA loci, namely MazEF, RelBE, and YefM-YoeB, were lately shown to be site-specific endoribonucleases (4, 7, 34). The recently elucidated structure of the FitAB complex from *Neisseria gonorrhoeae* indicated that the FitB toxin is composed of a PIN (PilT N terminus) domain, which is different from that of the RelE and YoeB toxins. However, FitB did not display nuclease activity against tested PIN substrates (35). The structures of PezT and zeta and subsequent mutagenesis experiments strongly infer the role of a phosphoryltransferase reaction in their toxicity. Notably, a recent report on the HipBA TA locus in *E. coli* indicated that the HipA toxin is a serine kinase that autophosphorylates itself in the presence of ATP (36). However, HipA is a member of the phosphotyrosinositol 3/4 kinase superfamily whose structure is not related to the NMP kinase fold of PezT and zeta. A common mechanism can therefore be excluded.

Expression of PezT and zeta in *E. coli* led to temporary inhibition in cell growth and is therefore seen as triggering cell stasis rather than cell death. However, expression of zeta in the Gram-positive *B. subtilis* was reported to be bactericidal (28). Consequently, there is a need to investigate the effects of PezT expression in Gram-positives such as *B. subtilis* and its native host, *S. pneumoniae*, to find out if PezT has a bactericidal effect in these organisms as described for zeta.

An earlier report had shown that the *pezAT* locus is part of a 27-kb pathogenicity island termed pneumococcal pathogenicity island 1 (PP11), which also harbors the iron uptake locus *piaABC* found to be required for full *S. pneumoniae* virulence in mice (37). A similar location of TA loci on mobile genetic elements has been previously reported (2, 9) for example, the presence of 13 TA loci in a 125-kb superintegron in *Vibrio cholerae* (9, 38). It was postulated that the presence of TA loci may contribute to the stability of these mobile elements similar to how plasmid-encoded TA loci function to stabilize plasmid replicons (2). In contrast to this, deletions were discovered in the genomes of many *S. pneumoniae* strains containing variable number of genes within PP11 and 33% of the isolates examined did not contain sp1051 (*i.e.* *pezT*) (39). Intriguingly, it was demonstrated that disruption of *pezT* impaired virulence in mouse models but showed no growth defect in laboratory broth, serum or blood. Although the function of the *pezAT* locus was not clear, the authors concluded that since *pezT* is missing in a proportion of strains, it is unlikely to be an essential gene for virulence, but rather may modulate the virulence of strains that carry it (39). A similar linkage between toxins of TA systems and virulence was postulated for the FitAB system of *N. gonorrhoeae*. *N. gonorrhoeae* lacks *fitAB* transit a polarized epithelial monolayer more quickly than the wild-type parent and has an accelerated rate of intracellular replication (35). The potential link between TA systems and virulence highlights the increasing importance for the study of TA systems in pathogenic bacteria where they have also been implicated in multidrug tolerance through their ability to mediate persistence.

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