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Structure of HrcQ_B-C, a conserved component of the bacterial type III secretion systems


*Department of Biology, University of Crete, P.O. Box 2208, GR-71409 Heraklion, Crete, Greece; †Institute of Molecular Biology and Biotechnology, P.O. Box 1527, GR-71110 Heraklion, Crete, Greece; ‡Astbury Centre for Structural Molecular Biology, School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, United Kingdom

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Type III secretion systems enable plant and animal bacterial pathogens to deliver virulence proteins into the cytosol of eukaryotic host cells, causing a broad spectrum of diseases including bacteremia, septicemia, typhoid fever, and bubonic plague in mammals, and localized lesions, systemic wilting, and blights in plants. In addition, type III secretion systems are also required for biogenesis of the bacterial flagellum. The HrcQ protein, a component of the secretion apparatus of Pseudomonas syringae with homologues in all type III systems, has a variable N-terminal and a conserved C-terminal domain (HrcQ_B-C). Here, we report the crystal structure of HrcQ_B-C and show that this domain retains the ability of the full-length protein to interact with other type III components. A 3D analysis of sequence conservation patterns reveals two clusters of residues potentially involved in protein–protein interactions. Based on the analogies between HrcQ_B and its flagellum homologues, we propose that HrcQ_B-C participates in the formation of a C-ring-like assembly.

In plant pathogens, the type III secretion system, termed Hrp, is required for pathogenicity on susceptible plants and is also essential for induction of a defense reaction called the hypersensitive response, on resistant plants (1–4). The Hrp system consists of a secretion apparatus encoded by ~25 hrp genes and its main function is to deliver virulence proteins (effectors) into plant cells (5–7). A broadly conserved subset of the hrp genes (designated hrc) appears to encode the core components of the delivery apparatus (2). It has been proposed that these core components are involved in the recognition of the secretion signals carried by the effectors and are thus responsible for the promiscuous character of type III systems for heterologous secreted proteins (8–10).

The HrcQ_B protein from Pseudomonas syringae belongs to the conserved core of the secretion apparatus of the type III system, with homologues in Erwinia, Xanthomonas,Ralstonia, and also in Yersinia, Shigella, Salmonella, and the flagellum (2). Notably, sequence similarity among the HrcQ_B homologues is restricted to their C-terminal regions (1). This conservation together with the variability of the N termini led to the proposal that these proteins may mediate interactions between the conserved and the species-specific components of type III systems (1).

The conserved C-terminal domain of HrcQ_B (HrcQ_B-C; residues 50–128) from P. syringae pv. phaseolicola was cloned in Escherichia coli and its structure was determined by x-ray crystallography. To our knowledge, this is the first structure reported for a conserved protein of the type III secretion apparatus.

Methods

Protein Expression and Data Collection. HrcQ_B-C comprises 84 amino acids, of which the first five have been introduced by the construct, and the remaining 79 correspond to residues 50–128 of the full-length protein (HrcQ2, SwissProt-ALL accession no. O85094). The expression, purification, and crystallization of HrcQ_B-C has been reported (11). For structure determination, Leu-72 was replaced by a methionine and the protein was expressed by the Met-auxotrophic E. coli strain B834 in the presence of selenomethionine. The anomalous dispersion of the incorporated selenium was exploited in a multiwavelength anomalous dispersion experiment. Diffraction data were collected at three wavelengths (Table 1) from a single, frozen crystal at the EMBL Hamburg Outstation (Deutsches Elektronen Synchrotron) and processed with the HKL package (12). A native data set collected at 2.3-Å resolution at the European Synchrotron Radiation Facility was used for the refinement.

Structure Determination and Refinement. The Se sites were identified by the program SOLVE (13). The experimental phases were improved by density modification techniques, including iterative noncrystallographic symmetry averaging and solvent flattening, and most of the model was AUTOBUILD and REBUILD by RESOLVE (14). Several rounds of refinement using CNS (15) and REFMAC5 (16) and manual rebuilding resulted in the final model (Table 1), which includes residues 55–126 of chain A, residues 55–125 of chains B and C, and residues 54–125 of chain D.

Multiple Sequence Alignment. Proteins used in the multiple sequence alignment shown in Fig. 3.4 are P. syringae pv. syringae HrpU (HrpU_ypr, SwissProt-ALL accession no. Q60235, 89% identity), Ralstonia solanacearum HrcQ (HrcQ_ral, Q52489, 33% identity), Yersinia pestis FliN (FliN_ypr, Q52489, 29% identity), Bradyrhizobium japonicum FliN (FliN_ypr, BAC52267, 33% identity), Salmonella typhimurium SpaO (SpaO_salen, Q56022, 26% identity), and Bordetella bronchiseptica BscQ (BscQ_ypr, Q0919E0, 32% identity).

Yeast Two-Hybrid System. The hrcQ_A gene was inserted into pAS2-1 plasmid to create an in-frame fusion with the GAL4 DNA-binding domain. The hrcQ_B gene was cloned in the pACT2 plasmid and fused with the transcription activation domain of GAL4. Plasmids were transformed into Saccharomyces cerevisiae strain PJ69-4A carrying three different GAL4-responsive reporter genes (ade, his, and lacZ), each driven by a different GAL4-responsive promoter, to reduce the incidence of false positives. The interaction of hybrid proteins was examined by growing the transformed cells on complete minimal medium lacking Trp, Leu, Ade, and His, for 3 days at 30°C. Plasmids pVLA3-1 and pTD1-1 are DNA-binding domain and activation

This paper was submitted directly (Track II) to the PNAS office.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.rcsb.org (PDB ID code 109Y).

A.P.T., N.J.P., and M.K. contributed equally to this work.

To whom correspondence should be addressed: University of Crete and Institute of Molecular Biology and Biotechnology–Foundation for Research and Technology, Hellas, P.O. Box 2208, Vassilika Vouton, GR-71409 Heraklion, Crete, Greece. E-mail: kokkinid@imbb.forth.gr.

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Table 1. Summary of crystallographic analysis

<table>
<thead>
<tr>
<th></th>
<th>Native data</th>
<th>Peak</th>
<th>Remote</th>
<th>Inflection</th>
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<tr>
<td>Unit cell dimensions</td>
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<tr>
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<td>BW7A (DESY)</td>
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<td>10,064</td>
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<td>94.5 (84.0)</td>
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<td>Mean figure of merit from experimental</td>
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<tr>
<td>Phasing</td>
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<td>Resolution, Å</td>
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<td>R-factor, %</td>
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<td>rmsd angles, °</td>
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</table>

Values in parentheses are for the highest-resolution shell. $R_{sym} = \frac{\sum_{i=1}^{I} \sum_{j=1}^{j} \sum_{h=1}^{H} I(h)}{\sum_{i=1}^{I} \sum_{j=1}^{j} \sum_{h=1}^{H} F_{o}}$ for the intensity i of j observations of reflection h. $R = \frac{\sum_{i=1}^{I} \sum_{j=1}^{j} \sum_{h=1}^{H} F_{o} - F_{c}}{\sum_{i=1}^{I} \sum_{j=1}^{j} \sum_{h=1}^{H} F_{o}}$ and $R_{max}$ is calculated for a randomly chosen subset of reflections that are omitted from refinement. rmsd, rms deviation.

Results and Discussion

Overview of the Structure. The crystal structure was determined by multiwavelength anomalous dispersion and refined to 2.3-Å resolution. Table 1 shows details of the data collection, processing, and refinement statistics. As shown in Fig. 1, A and B, HrcQαC is an elongated, gently curved homotetramer with approximate dimensions of 25 × 30 × 90 Å. The four monomers assemble into two tightly bound homodimers that are packed together to form a dimer of dimers. The dimers associate with each other in such a way that the angle between their major axes of inertia is ~120°. The secondary structure of each monomer consists of five β-strands (labeled β1-β5 in Fig. 1) and one short helix (labeled α1). Two monomers (A and B or C and D chains in Fig. 1C) fold together in a symmetrical manner to form a compact and intertwined dimeric structure that comprises two six-stranded antiparallel β-sheets bridged through a long β-ribbon (β1-β1 in Fig. 1A) that spans the length of the whole dimer and is shared by the β-sheets (Fig. 2). The β-ribbon connects the monomers through an extended array of hydrogen bonds and is further stabilized by a disulfide bond between the symmetry related cysteines 67 (Fig. 1C). This covalent bond (being completely buried in the interior of the molecule) remains intact in the crystals despite the presence of reducing agent (10 mM dithiothreitol). Strands β3, β4, and β5 of monomer A and strand β2 of B (Fig. 1A) are successively hydrogen bonded and together with the first half of the β-ribbon form the complete six-stranded β-sheet.

The dimer–dimer interface consists of a six-stranded antiparallel β-barrel formed by the β2 strands of chains B and C and the β4, β5 strands of chains A and D (Fig. 1A and B). A total surface area of 1,228 Å² is buried on tetramer formation through dimer association. In addition, the tetramer is stabilized by hydrogen bonds formed between carbonyl and amino groups of the protein backbone at the positions of Ile-85 of B and C chains and Asp-112 and Glu-114 of domain fusion plasmids that provide a positive control for interacting proteins.

Immunoblotting and Far Western Blot Analysis. Purified proteins were electrophoresed through SDS-polyacrylamide gel and transferred to poly(vinylidene difluoride) (PVDF) membrane. For immunoblotting, the membrane was blocked in a solution of Tris-buffered saline/0.1% Tween 20/5% nonfat milk, incubated with the appropriate antibodies and the signal was detected by nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrates of AP-conjugate. For Far Western blot analysis, the membrane was immersed with 8 M urea in 20 mM Tris-HCl, pH 7.5/60 mM NaCl/10 mM MgCl₂/0.1 mM EDTA/5% glycerol/0.02% Nonidet P-40. Proteins absorbed to the membrane were renatured and the membrane was blocked as described above and incubated overnight at 4°C with [35S]-labeled protein. The signal was detected by autoradiography. Rabbit polyclonal antisera specific for HrcQα and HrcQβ were used for the immunoblotting assays.

Subcellular Fractionation Experiment. P. syringae pv. phaseolicola NPS3121 cells were grown for 14 h in hrp-inducing medium and separated into culture supernatant and cell-bound fraction by centrifugation at 10,000 × g. The supernatant was passed through a 0.22-μm filter and concentrated by centricon-10 (Amicon). Both fractions were analyzed by SDS/PAGE and immunoblotting. The cell-bound fraction was further fractionated into a cytoplasmic and a total membrane fraction by ultracentrifugation at 65,000 × g for 40 min. The total membrane fraction was mixed with urea or NaCl or Triton X-114 and incubated on ice for 30 min before ultracentrifugation at 65,000 × g for 40 min. Both the supernatant and the pellet were analyzed by SDS/PAGE, followed by immunoblot analysis.
A and D. The Stokes’ radius of the protein in solution, which was estimated by size exclusion chromatography to be \( \equiv 28.5 \) Å, is in excellent agreement with the value of 29.1 Å as calculated (17) from the crystal structure of the whole tetramer.

Sequence Conservations and Identification of Functional Residues.

HrcQ_{B-C} shares a sequence identity ranging between 33% and 89% with the C termini of homologues from other phytopathogenic bacteria. It also has an identity between 29% to 33% with the flagellar FliN proteins and between 28% to 32% with homologues from animal pathogens. A multiple sequence alignment of these homologues reveals well conserved individual amino acids and extended regions of high similarity (Fig. 3A). Four residues, Leu-77, Gly-108, Val-111, and Gly-118, are strictly conserved among all aligned sequences. Glycines, in particular, located on strands \( \beta 4 \) and \( \beta 5 \), respectively, are completely buried into the hydrophobic core of the HrcQ_{B-C}, in a tightly packed region where there is no space for side chains to be accommodated. The great majority of the other conserved residues are

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Fig. 1. A schematic representation of the HrcQ_{B-C} structure. Each chain is individually colored and labeled. (A) A view of the complete tetramer. The dimers are related by a two-fold axis that is indicated by a red line. (B) A view of the tetramer along the two-fold axis. The molecule is rotated 90° relative to A. (C) A stereoview of the HrcQ_{B-C} dimer. The red line indicates the local two-fold axis. Symmetry related cysteines that form the disulfide bond are shown as a ball-and-stick model.

Fig. 2. A stereoview of the \( 2F_o - F_c \) electron density map contoured at 1.5 \( \sigma \) with the final model overlaid on it. The region shown corresponds to part of the sheet formed by the strands \( \beta 1 \) (chain A), \( \beta 1 \) (chain B), and \( \beta 3 \) (chain B).
Interactions of HrcQb-C with Other Type III Components. It has been previously shown by electron microscopy that the type III secretion apparatus is a supramolecular structure (21). In this context, and considering the indications for the existence of protein–protein interfaces, we investigated potential interactions between the HrcQb and other type III components, through genetic (yeast two-hybrid system) and biochemical (Far Western and immunoprecipitation) experiments.

Our studies demonstrate that in the P. syringae secretion apparatus: (i) HrcQb strongly binds to HrcQA, a protein encoded by a gene immediately upstream of hrcQb (Fig. 5A), (ii) HrcQb-C retains the ability of the full-length protein to interact with HrcQA (Fig. 5B), and (iii) only the C terminus (residues 66–238) of HrcQb is involved in the association with HrcQA (Fig. 5C and D). The HrcQb–HrcQA interaction is consistent with the observation that these proteins are expressed as a single product, named HrcQ (22), in the genera Erwinia, Xanthomonas, and Ralstonia. We have also performed subcellular localization experiments (Fig. 6) that show that neither HrcQb nor HrcQA are found in the culture supernatant of P. syringae cells, indicating that they are not secreted components. The same experiments showed that HrcQb fractionates in both cytoplasmic and membrane extracts suggesting that it is cytoplasmic or loosely associated with a membrane component. In addition, HrcQb is exclusively found in the membrane fraction; this finding is consistent with the presence of a transmembrane domain that is predicted on the basis of sequence data (data not shown). Our results are fully consistent with earlier findings for flagellar system: (i) the FlIN/FlIM proteins, which are the flagellum counterparts of HrcQb/HrcQA, interact with each other (23–26), (ii) the C terminus of the FlIN protein (which is homologous to HrcQb-C) is sufficient for both flagellar assembly and function (24), (iii) the C terminus of FlIM is sufficient for binding FlIN.
participate.

In which, most likely, the C-terminal parts of both proteins of a multiprotein, ring-shaped assembly, known as C-ring (23–25), in which fractionates from the membrane. In addition, it has been shown that FliN–FliM, it has been suggested that the two proteins might share some common structural features that participate in the formation of the C-ring, possibly by means of the occupation of quasiequivalent positions (25). Consistent with this hypothesis, we also observe a significant homology (20.5% identity and 54.5% similarity; Fig. 3B) between the HrcQα–C and the C-terminal segment of HrcQα with a concomitant agreement on the predicted (29) secondary structure (data not shown). A complete coincidence is found between the predicted and experimentally determined secondary structural elements for residues 170–185 of HrcQα and the homologous HrcQβ-C segment (residues 65–80), respectively; this HrcQβ-C segment contains a cluster of residues possibly involved in protein–protein interfaces (Fig. 4).

This structure determination of a component of the bacterial type III secretion apparatus, highly conserved among plant, animal, and human pathogens, has broad implications in the understanding at an atomic level of bacterial pathogenesis and in drug design.

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