A Periplasmic Complex of the Nitrite Reductase NirS, the Chaperone DnaK and the Flagellum Protein FliC is essential for Flagellum Assembly and Motility in Pseudomonas aeruginosa

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ABSTRACT

_Pseudomonas aeruginosa_ is a ubiquitously occurring environmental bacterium and opportunistic pathogen responsible for various acute and chronic infections. Obviously, anaerobic energy generation via denitrification contributes to its ecological success. To investigate the structural basis for the interconnection of the denitrification machinery to other essential cellular processes we have sought to identify the protein interaction partners of the denitrification enzyme nitrite reductase NirS in the periplasm. We employed NirS as an affinity purifiable bait to identify interacting proteins _in vivo_. Results obtained revealed that both the flagella structural protein FliC and the protein chaperone DnaK form a complex with NirS in the periplasm. The interacting domains of NirS and FliC were tentatively identified. The NirS interacting stretch of amino acids lies within its cytochrome c domain. Motility assays and ultrastructure analyses revealed that a nirS mutant was defective in the formation of flagella and correspondingly in swimming motility. In contrast, the fliC mutant revealed an intact denitrification pathway. However, deletion of the nirF gene, coding for a heme d1 biosynthetic enzyme which lead to catalytically inactive NirS, did not abolish swimming ability. This pointed towards a structural function for the NirS protein. FliC and NirS were found co-localized with DnaK at the cell surface of _P. aeruginosa_. A function of the detected periplasmic NirS-DnaK-FliC complex in flagella formation and motility was concluded and discussed.
IMPORTANCE

Physiological functions in Gram-negative bacteria are connected with the cellular compartment of the periplasm and its membranes. Central enzymatic steps of anaerobic energy generation and the motility mediated by flagella activity are using these cellular structures besides multiple other processes. Almost nothing is known about the protein network functionally connecting these processes in the periplasm. Here, we demonstrate the existence of a ternary complex consisting of the denitrifying enzyme NirS, the chaperone DnaK and the flagellar protein FliC in the periplasm of the pathogenic bacterium P. aeruginosa. The dependence of flagellum formation and motility on the presence of an intact NirS was shown, structurally connecting both cellular processes, which are important for biofilm formation and pathogenicity of the bacterium.

INTRODUCTION

Pseudomonas aeruginosa is a metabolically versatile bacterium inhabiting multiple environmental niches (1). It is known for its highly efficient growth in the absence of oxygen. Fast anaerobic growth is mediated via the utilization of different N-oxides as electron acceptors in the respiratory chains of denitrification (2). Anaerobic respiratory growth via denitrification also sustains biofilm formation on environmental surfaces, in the mucus in the lung of cystic fibrosis patients, on the epithelium of the urinary tract infected individuals and in burn wounds (3).

The periplasm – the cellular compartment bounded by the cytoplasmic membrane and the outer membrane of Gram-negative bacteria – is a unique
compartment, rich in signal transduction and transport systems, and other connections between the outer cell surface and the cytoplasm. It selectively links and buffers external and internal environments, and connects systems that monitor external parameters with internal systems that respond to such parameters. It is the “telephone exchange” and logistics central of the cell envelope. It also orchestrates cellular actions directed at the external environment, including the attachment to and attack of host cells by infecting pathogens. Present knowledge of periplasm structure and function is patchy, and a deeper understanding of bacterial behavior in environmental settings, and in particular of bacterial activities related to infections, will inter alia depend upon new advances in periplasm biology.

In the process of denitrification nitrate is used as terminal electron acceptor and is reduced to N\textsubscript{2} in four steps catalyzed by nitrate (Nar), nitrite (Nir), nitric oxide (Nor) and nitrous oxide (Nos) reductases, respectively (1, 4-6). Except for the first reduction catalyzed by the Nar enzyme, all these reactions are carried out in the bacterial periplasm. The periplasmic denitrification pathway is intimately connected with other central cellular processes including respiratory energy generation, transmembrane transport, protein translocation, environmentally controlled gene regulation, disulfide bond formation, flagellum assembly and function, the biogenesis of cytochrome c, heme d\textsubscript{1} (2) and various processes important to pathogenesis. Of special interest is the \textit{P. aeruginosa} cytochrome \textit{cd}\textsubscript{1} nitrite reductase (NirS), a homodimer composed of two distinct domains. The N-terminal domain is a \textit{c}-type cytochrome accommodating a covalently bound heme \textit{c}, while the C-terminal domain harbors the catalytic heme \textit{d}\textsubscript{1} molecule (7, 8). This enzyme catalyzes the reduction of nitrite to nitric oxide.
Interestingly, a nirS knockout mutant of *P. aeruginosa* PA14 showed deficiency in swarming motility (9) which, in *P. aeruginosa*, involves both flagella and pili, unlike swimming motility which is accomplished exclusively by flagella (10). The flagellum, in combination with the chemotaxis system, mediates directional movement of the bacterium in response to attractants and repellents (10), and plays a crucial role in initial cell-cell and cell-surface interactions including biofilm formation (11). A nirS mutant produces poorly-dispersing biofilms, which partially regain dispersal ability upon addition of exogenous nitric oxide (12, 13).

These observations suggest a coupling between denitrification and motility, though direct evidence for this was lacking.

Though small signaling molecules are involved in the coupling of many cellular processes, physical protein-protein interactions are equally important in orchestrating metabolic and regulatory networks. They are expected to be particularly relevant for the control of biochemical processes in the periplasm.

In this study we have sought the interaction partners of NirS, through application of interactomic methods, phenotypic characterizations and electron microscopy-based imaging. This has revealed the existence of a periplasmic protein interaction triad composed of NirS, the flagellar protein FliC and, surprisingly for a protein previously thought to be cytoplasmic, the molecular chaperone DnaK. This complex connecting the denitrification machinery with motility via flagellum assembly might be only the beginning for the understanding of complex dynamic protein-protein interactions in the periplasmic compartment.

**MATERIALS AND METHODS**
Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Tables 1 and A1. *Escherichia coli* DH10b (ThermoFisher Scientific, Waltham, USA) was employed as host propagator of the constructs obtained in this study. *E. coli* BL21 was used to overproduce DnaK for polyclonal antibody generation. *P. aeruginosa* PA14 wild-type strain (14) and several PA14 MAR2xT7 library mutants (15) were used throughout this study. The pAS40 vector was utilized to express nirS, nosZ and fliC by their natural promoters in *P. aeruginosa*. The pJET1.2 plasmid (ThermoFisher Scientific, Waltham, USA) served as intermediate cloning vector carrying the promoter-gene insert, prior to its final cloning into pAS40 for the fusion of the target proteins with Strep-tag II. The pET14b plasmid was used to produce DnaK-His$_{6x}$ protein for polyclonal antibody production (Metabion AG, Planegg/Steinkirchen, Germany). *E. coli* was transformed by the heat shock method (16) and *P. aeruginosa* by electroporation (17). Plasmid preparations were carried out according to the manufacturer’s guidelines (QIAGEN Miniprep Kit). Molecular DNA techniques were performed as formerly described (18). DNA sequencing of the constructs was performed by GATC Biotech AG (Konstanz, Germany). All enzymes used during the study were obtained from New England Biolabs GmbH (Frankfurt am Main, Germany).

For aerobic growth, all strains were grown in Luria Bertani (LB) medium in shake flasks aerated by rotation at 180 rpm. For anaerobic growth, strains were grown until late exponential phase in LB supplemented with 50 mM nitrate in rubber-stoppered serum flasks rotated at 100 rpm (19). Where specified, *P. aeruginosa* strains were cultivated in swimming medium (20) supplemented with 20 mM arginine aerobically until an optical density of 1.0 at 578 nm was
reached and immediately shifted into anaerobic flasks and further incubated for 5 days. Incubation temperature was 37°C. All experiments were done in triplicate. Required antibiotics were added at the following concentrations (μg/mL): Ampicillin (Ap), 100; Carbenicillin (Cb), 100 for *E. coli* DH10b and 250 for *P. aeruginosa*.

**Construction of *P. aeruginosa* strains for tagged protein complex formation.** Construction of the plasmid carrying the target genes (*nirS*, *fliC* and *nosZ*) under the control of their natural promoters, but fused to the DNA encoding Strep-tagII was achieved using a slightly modified Golden Gate cloning protocol. This cloning strategy is shown in Figs. A1 and A2. During the procedure restriction endonuclease *Bsa*I recognizes the DNA sequence GGTCTC and cleaves 1 nucleotide upstream the same strand and 5 nucleotides in the complementary strand, leaving a sticky end of 4 nucleotides which was customized. Two single-strand DNA sequences (primers, 5´ to 3´) were annealed. The forward primer consisted of: an *EcoRI* restriction site, 4 first nucleotides of the cloning region (~400 - 500 bp upstream the gene of interest to cover the promoter region), one random nucleotide, the restriction sites for *Bsa*I (inversed orientation: GAGACC), *Bgl*II, *EcoRV*, *Bsa*I (GGTCTC), one random nucleotide, the last 4 nucleotides of the gene of interest (stop codon omitted), the DNA encoding the Strep-tag II and a stop codon (TCA). The reverse primer was the forward complementary strand lacking the *EcoRI* restriction site at the 5´ and including instead a *Hind*III restriction site at its 3´. After the fragment was annealed, it was ligated into the *EcoRI*-HindIII digested pAS40 plasmid. The fragments cloned were *nirS*StreptagIIFw-*nirS*StreptagIIRv, *fliC*StreptagIIFw-*fliC*StreptagIIRv and *nosZ*StreptagIIFw-*nosZ*StreptagIIRv.
(Table A2). The BglII and EcoRV digestion sites were employed to confirm proper cloning. In parallel, nirS and fliC genes along with approximately 400 - 500 bp upstream (including promoter regions) (21, 22) were amplified and further cloned into the plasmid pJET1.2. Both genes included one BsaI (GGTCTC) restriction site at the 5´ and 3´ extremes. The nosZ gene and corresponding nos promoter (approximately 400 - 500 bp upstream of nosR) (23) were amplified separately and cloned into pJET1.2. Afterwards, both constructed plasmids pAS40 and pJET1.2 were mixed and subjected to 50 cycles of 5 min BsaI digestion followed by 5 min ligation. This way, the pJET1.2 constructed vectors were continuously subjected to digestion with a release of the genes of interest, whereas once the genes were inserted into pAS40, resulting constructs were stable due to the loss of BsaI restriction sites. This cloning approach resulted in a high yield of pAS40 plasmids harboring the genes of interest fused to the Strep-tag II at the C-terminus. Five strains were obtained following this strategy, expressing nirS, fliC and nosZ in three different mutant backgrounds. They are listed in Table 1.

Isolation of the periplasmic fraction. P. aeruginosa strains grown anaerobically to the exponential phase were harvested by centrifugation at 4,000 g, 4°C for 20 min. Resulting pellets were washed twice in PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4 x 7 H2O and 1.4 mM KH2PO4) and the isolation of the periplasmic contents was performed following a previously described method with minor changes (24). Washed cells were resuspended in PE buffer (20% w/v sucrose, 50 mM Tris pH 8.0 and 1 mg/mL polymyxin B) supplemented with 1 tablet/10 mL of protease inhibitor cocktail (Roche Diagnostics, Berlin, Germany). Cell suspensions were incubated overnight at
4°C with continuous mixing, followed by centrifugation at 100,000 g for 1 h and 4°C in an Optima L-90K ultracentrifuge (Beckman Coulter, Krefeld, Germany) with a 70.1 Ti rotor (Beckman Coulter, Krefeld, Germany). The resulting supernatant fluid constituted the periplasmic fraction.

In vivo crosslinking. Where indicated, native protein assemblies were stabilized prior to periplasmic extraction by in vivo crosslinking, performed as formerly described (25). The introduction of covalent bonds between interacting proteins enabled more stringent washing reactions and reduces background by largely conserving the native protein ratios in a multiprotein assembly. Formaldehyde, which mediates very specific crosslinking between proteins in close proximity (26), was injected by syringe into the anaerobic cultures to a final concentration of 0.125% w/v. The crosslinking reaction was carried out by incubation at 150 rpm for 20 min at 37°C, to enable permeation through cell membranes and was terminated by injecting glycine to a final concentration of 130 mM, and incubation for a further 5 min with shaking. Protein samples were then heated to 95°C for 20 - 30 min, prior to analysis by SDS-gel electrophoresis (27).

Affinity co-purification of the bait proteins and their interaction partners. Periplasmic fractions were subjected to affinity purification by applying the samples to gravity-flow column of Strep-Tactin® superfloss high capacity (IBA GmbH, Göttingen, Germany). Washing and elution were carried out according to the manufacturer’s guidelines. A periplasmic extract of P. aeruginosa PA14 wild-type strain harboring the parental plasmid was used as background control. Protein concentrations of the elutate fractions were determined by means of the
Bradford assay (28) and eluates between 0.05 - 0.2 mg/mL were analyzed either by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or liquid chromatography tandem-mass spectrometry LC-MS/MS. For SDS-PAGE analyses, eluted fractions were precipitated by adding 50% v/v trichloroacetic acid (TCA), 0.1% w/v sodium desoxycholate to the protein sample (ratio of 2.5:9:1) and subsequently incubated on ice for 30 min. Precipitates were collected by centrifugation at 13,000 rpm for 20 min and washed twice with cold acetone, followed by centrifugation for 10 min at 13,000 rpm and 4°C. Precipitates were resuspended in 20 µL SDS-loading buffer (15% v/v glycerol, 5% v/v beta-mercaptoethanol, 2.4% w/v SDS, 1% w/v bromophenol blue, 0.8% w/v Tris, pH 6.8), heated at 95°C for 20 - 30 min and subsequently loaded on a 12.5% SDS-gel (29).

**Antibody generation.** Specific polyclonal antibodies were obtained against overproduced DnaK protein and synthetic peptides from NirS and FliC. For this purpose DnaK-His$_{6x}$ was produced in *E. coli* BL21 using the pET14b plasmid. Purification of approximately 2 mg/mL recombinant protein was achieved following the manufacturer’s guidelines (Protino, Macherey Nagel, Düren, Germany). Polyclonal rabbit antibodies were generated by Metabion International AG (Planegg/Steinkirchen, Germany). NirS peptides covering the regions from amino acid 379 to 392, 526 to 540 and 541 to 555 were synthesized and the mixture was employed for raising polyclonal rabbit antibodies. For the generation of anti-FliC antibodies, a peptide consisting of the first 174 amino acids of FliC was used for rabbit immunization. IgGs were purified on a sepharose protein A column.
Co-localization experiments using transmission electron microscopy. *P. aeruginosa* PA14 was grown anaerobically in LB supplemented with 50 mM nitrate until late exponential phase (8 h) and fixed with 1% formaldehyde in growth medium overnight at 5°C. After centrifugation at 4,000 g for 20 min at 5°C, harvested bacteria were resuspended in TE buffer containing 10 mM glycine to quench free aldehyde groups. Bacteria were then dehydrated according to the progressive lowering of temperature protocol (PLT embedding) using ethanol, and embedded in Lowicryl K4M resin. After polymerization with UV-light, ultrathin sections were cut with a diamond knife, collected onto butvar-coated nickel grids and incubated with the specific antibodies. First, sections were incubated with a 1:25 dilution of the purified DnaK IgG antibodies overnight at 5°C. After washing with PBS, bound antibodies were made visible by incubating with protein A/G-conjugated with gold nanoparticles (PAG) 15 nm in size (1:75 dilution of the stock solution) for 30 min at room temperature. After washing with PBS containing 0.1% Tween, 100 sections were incubated with a protein A solution (0.1 mg/mL) for 15 min at room temperature. Sections were incubated either with anti-NirS or anti-FliC antibodies (1:20 dilution of the purified IgG) for 3 h at room temperature. After PBS washing, sections were incubated with PAG (10 nm in size, 1:200 dilution of the stock solution) for 30 min at room temperature. Subsequently, sections were washed with PBS containing 0.1% Tween 100, TE-buffer and distilled water. Sections were counter-stained with 4% aqueous uranyl acetate for 1 min. Samples were then examined in a TEM910 transmission electron microscope (Carl Zeiss, Oberkochen, Germany) at an acceleration voltage of 80 kV. Images were recorded digitally at calibrated magnifications with a Slow-Scan CCD-Camera.
(ProScan, 1024x1024, Scheuring, Germany) with ITEM-Software (Olympus Soft Imaging Solutions, Münster, Germany). Contrast and brightness were adjusted with Adobe Photoshop CS4.

**Immunofluorescence microscopy.** For immunofluorescence staining of flagella, bacteria were grown aerobically in swimming medium or LB supplemented with 20 mM arginine to an OD of 1.0 and then switched to anaerobic growth (as described before) for a period of 5 days. Samples were taken and fixed with 3% paraformaldehyde in PBS for 30 min at room temperature. No washing and centrifugation steps were performed to avoid possible damage of the flagellar structures. A drop (25 µL) of bacterial culture was placed on a glass poly-L-lysine coated coverslip. Fixed coverslips were rinsed twice after each immunostaining step with PBS. Samples were treated with blocking buffer (10% FCS in PBS) for 1 h at room temperature. Coverslips were incubated with the anti-FliC primary rabbit polyclonal antibody in blocking buffer for 1 h at room temperature. After washing, samples were incubated with the fluorescently-labeled secondary antibody goat anti-rabbit Alexa 488 (Molecular Probes, Eugene, Oregon, USA). Coverslips were mounted using ProLong Gold containing nuclear staining (DAPI, Molecular Probes). Fluorescence images were obtained with an Axio Imager A1, AxioCam MRm camara (Zeiss, Oberkochen, Germany) and AxioVision Rel. 4.6 Software using AxioVision 4 Module Multichannel Fluorescence and Filters 49 and 44.

**SDS polyacrylamide gel electrophoresis and western blot detection.** Bait proteins and interaction partners were separated by SDS-PAGE, stained in a solution of 0.05% w/v Coomassie Brilliant Blue GS-250 in 25% v/v isopropanol.
and 10% v/v acetic acid and visualized under a GS-800 calibrated densitometer (BioRad, Munich, Germany). Interactions involving proteins NirS and FliC were corroborated by Western blot analysis. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and detected with Strep-Tactin-conjugated alkaline-phosphatase, according to the procedure recommended by the supplier (IBA GmbH, Göttingen, Germany).

**LC-MS/MS and data analyses.** Prominent proteins were excised from the polyacrylamide gels and prepared for LC-MS/MS analysis (30). Protein samples were supplemented with 1 µL GlycoBlue (Ambion, Darmstadt, Germany), 80 µL 2.5 M Na acetate pH 5.0 and 1.5 mL ethanol, and incubated overnight at room temperature to precipitate the solved proteins. The precipitated proteins were pelleted (16,000 rpm, 30 min, 4°C) and air dried. Air dried proteins were resolubilized in 40 µl of 50 mM triethylammonium bicarbonate buffer (TEAB), reduced by addition of 4 µl of 20 mM Tris-(2-carboxyethyl)-phosphine-hydrochloride (TCEP), alkylated with 2 µl of 200 mM methyl methane thiosulfonate (MMTS), digested by the addition trypsin to a final ratio of 50:1 (protein:protease), and incubated at 37°C overnight. After evaporation of all fluid in a SpeedVac (Eppendorf, Hamburg, Germany), the peptides were desalted using Stage Tips with an additional layer of 5 µL of 10 µm LiChrosorb® RP-18 (Merck Millipore, Darmstadt, Germany) material (Lichrosorb; Merck Millipore, Darmstadt, Germany) as previously described (31). Peptides were resolubilized in 20 µl of 3% acetonitrile (ACN) containing 0.2% trifluoroacetic acid (TFA) and adsorbed to the RP18 material. After washing with resolubilization solution, the peptides were eluted with 60% ACN containing 0.2% TFA. To remove the
organic phase the peptides were dried in a SpeedVac and resolubilized in 12 µL of 3% ACN containing 0.2% TFA.

LC-MS/MS analyses were performed on a Dionex UltiMate 3000 n-RSLC system connected to an LTQ Orbitrap Velos mass spectrometer (ThermoFisher Scientific, Waltham, USA). Peptides were loaded onto a C18 pre-column (3 µm, Acclaim, 75 µm x 20 mm, Dionex, ThermoFisher Scientific, Waltham, USA) in 3% ACN containing 0.1% TFA washed for 3 min in 3% ACN containing 0.1% formic acid (FA) at a flow rate of 6 µl/min, and subsequently fractionated on a C18 analytical column (3-µm, Acclaim PepMap RSLC, 75 µm x 25 cm, Dionex) at 350 nl/min with a linear 120 min gradient of 0 - 80% ACN in 0.1% FA. The LC system was operated with Chromeleon Software (version 6.8, Dionex), which was embedded in the Xcalibur software (version 2.1, ThermoFisher Scientific, Waltham, USA). The effluent from the column was electro-sprayed (Pico Tip Emitter Needles, New Objectives, Woburn, USA) into the mass spectrometer controlled by Xcalibur software and operated in the data-dependent mode allowing the automatic selection of a maximum of 10 doubly and triply charged peptides and their subsequent fragmentation. Peptide fragmentation was carried out using the CID mode in the ion trap. MS/MS raw data files were processed via Proteome Discoverer 1.3.0.339 mediated searches against a P. aeruginosa PA14 database, extracted from SwissProt on a Mascot server (V. 2.4, Matrix Science, Boston, Massachusetts, USA). The following search parameters were used: enzyme, trypsin; maximum missed cleavages, 1; fixed modification, carbamidomethylation (C); variable modification, oxidation (M), peptide tolerance, 5 ppm; MS/MS tolerance, 0.5 Da.
Analysis of the peptides involved in the NirS-FliC interaction. To investigate the peptides involved in the interaction between NirS and FliC, eluates obtained from the affinity chromatography of all free extracts from PA14 nirS⁻/nirS⁺, PA14 fliC⁻/nirS⁺ and PA14 nirS⁻/fliC⁺, were digested by trypsin and analyzed by LC-MS/MS for complete peptide composition analysis. A schematic representation of the strategy followed is shown in Fig A3. The NirS peptides involved in the interaction with FliC were superimposed on an existing protein model (pdb accession code 1NIR) (32), using the PyMOL Molecular Graphics System, Version 1.5.04 Schrödinger L.L.C. (33).

Swimming assay. Swimming motility was tested on swimming medium as described before (20). Petri dishes were prepared with 1% w/v tryptone, 0.5% NaCl, 0.3% w/v agarose medium containing 20 mM L-arginine as energy source. The plates were point inoculated in the middle by stabbing with an overnight culture tip-soaked toothpick and placed in an Anaerocult anaerobic incubation container with Anaerocult A sachet (Merck Millipore, Darmstadt, Germany). Oxygen depletion was confirmed colorimetrically by an Anaerotest indicator strip (Merck Millipore). The plates were incubated bottom up at 37°C for 5 days. Swimming motility was determined by measuring the colony halo diameter.

RESULTS

The NirS, FliC and DnaK form a complex in the periplasm of P. aeruginosa grown under denitrifying conditions. In order to investigate the relationship of anaerobic energy generation via the periplasmic nitrite reductase NirS and
FliC containing flagellum mediated motility, physical protein-protein interactions studies were performed under anaerobic denitrifying conditions. Firstly, for this purpose the interaction partners of the periplasmic cytochrome $cd_1$ nitrite reductase NirS were investigated in a Strep-Protein Interaction Experiment (SPINE) (34), with NirS as a “bait” protein. The nirS gene and approximately 500 bp of its upstream sequence were cloned into the P. aeruginosa plasmid pAS40. Thus, nirS expression was controlled by the native transcriptional regulation machinery under denitrifying conditions (35). The plasmid was introduced into the PA14 nirS$^-$ mutant strain to yield the PA14 nirS$^-$/nirS$^+$ complemented strain. Samples of growing bacteria were taken from cultures incubated under anaerobic denitrifying conditions, treated or not (control) with crosslinker. Periplasmic extracts were prepared and subjected to affinity purification of the resulting NirS-protein complexes. SDS-PAGE analyses of these NirS-protein complexes eluted from the column revealed two distinct protein species, in addition to the NirS bait protein. The identity of the purified bait Strep-tagged NirS was confirmed by western blotting (Fig. 1A). The other two proteins were always present in the experiments performed in triplicates (Fig. 1A). These co-purifying proteins were identified as FliC (flagellin type B) and the chaperone DnaK by LC-MS/MS analyses. A reciprocal experiment with FliC as the bait protein produced in the PA14 fliC$^-$ mutant background (PA14 fliC$^-$/fliC$^+$) confirmed the initial observation. In this case, NirS and DnaK were found bound and co-purifying with FliC (Fig. 1B). Other co-eluting proteins were identified as multiple subunits of the flagellum (data not shown). The NirS-FliC-DnaK triad was detected in samples from both in vivo crosslinked cultures and untreated cultures, indicating that the observed interactions were rather stable,
although the yields of co-purifying proteins were substantively lower from cultures that had not been treated with crosslinker (Fig. 1A). No column-binding proteins were observed with periplasmic samples from control cells of the wild-type PA14 strain bearing the cloning vector pAS40 alone (Fig. 1C). In a further control involving another periplasmic denitrification pathway enzyme, the nitrous oxide reductase NosZ, as a bait protein, the FliC and DnaK were not found bound to the enzyme, which underlines the specificity of the NirS-FliC-DnaK interactions (data not shown).

A *P. aeruginosa* nirS mutant is deficient in swimming motility. To investigate the relevance of enzymes of the denitrification pathway for *P. aeruginosa*, the swimming motility of mutant strains defective in different denitrification reductases was tested in appropriate motility assays. *P. aeruginosa* PA14 wild-type and a corresponding *fliC* mutant strain served as positive and negative controls, respectively. Because denitrification mutants fail to grow anaerobically with nitrate as electron acceptor, the fermentable substrate arginine was provided in the cultures to enable anaerobic growth of the mutants. Under these conditions, any swimming defect should be related to a malfunction of the flagellum rather than to impaired denitrification. As shown in Fig. 2, the *P. aeruginosa* PA14 nirS*" mutant was clearly impaired in its swimming ability. The degree was comparable to that of the *fliC* mutant strain. The nirS*" complemented strain *P. aeruginosa* PA14 nirS*"/nirS*+, and the fliC*" complemented strain *P. aeruginosa* PA14 fliC*"/fliC*+ both exhibited normal motility, i.e. the characteristic large swimming halo of the wild-type parental strain (Fig. 2). Most importantly, the *P. aeruginosa* PA14 narH and nosZ mutant strains were also able to swim, which demonstrated that denitrification
per se is not crucial for *P. aeruginosa* motility. Thus, the involvement of the
denitrification machinery in motility appears to be NirS-specific. In order to
distinguish between a poorly structural and a catalytically, i.e. energy-delivering
function of NirS, a catalytically inactive NirS was tested. For this purpose a *P.
aeruginosa nirF* mutant, defective in the biosynthesis of the essential heme d1
cofactor of nitrite reductase was analyzed for its swimming behavior (25). The
*nirF* was described to produce catalytically inactive but stable NirS in the
periplasm (36). Clearly, the swimming activity observed for the *nirF* mutant,
carrying a catalytically inactive NirS, pointed towards a structural function of the
NirS protein in the triple complex. In agreement, the functional consequences of
the NirS-FliC interaction were not reciprocal, i.e. the *nirS* mutant did not swim
whereas the denitrifying growth of the *fliC* mutant was not affected (Fig. A4).
The anaerobic growth and motility defect of the *P. aeruginosa PA14 norB*–
mutant is most likely caused by the previously described essential role of NorB
in the formation of catalytically active NirS (37) (Fig. 2). Finally, testing of the
*nirS* mutant for swimming under aerobic growth conditions revealed a partial
restoration of swimming ability. Approximately 40% of the analyzed strains
showed the restored phenotype. It looked like we observed of mixture of strains
in either their aerobic and anaerobic mode. However, de la Fuente et al.
showed the swarming motility defect in *P. aeruginosa* for a *nirS* mutant under
aerobic conditions (9). Obviously, further research is needed to clarify the
structural basis of aerobic versus anaerobic flagellum assembly.

*P. aeruginosa PA14 nirS*– mutant was impaired in flagella assembly. In
order to investigate the functional basis for the observed motility defect of the *P.
aeruginosa PA14 nirS*– mutant, the flagellation of this mutant strain was tested
by immunofluorescence microscopy using specific antibodies against FliC. The flagella abundance and morphology of \textit{P. aeruginosa} wild-type and \textit{nirS} \textsuperscript{-} mutant were compared. The wild-type parental strain produced flagella under aerobic and anaerobic growth conditions, when grown in LB, although flagellar abundance varied from cell to cell (Fig. 3).

The number of flagellated \textit{P. aeruginosa} PA14 \textit{nirS} \textsuperscript{-} mutant bacteria was significantly lower compared to the parental strain. Many short, incomplete and abnormal non-spiral (non-rotating) flagella were observed (Fig. 3, arrow heads).

Similar results were obtained with bacteria cultured in so-called swimming medium. These results demonstrated that the NirS protein is functionally required for correct flagellum functionality and as a consequence for motility in \textit{P. aeruginosa}.

**Immunolocalization of NirS-FliC-DnaK.** In order to directly visualize the spatial distribution of NirS, FliC and DnaK in cells, purified specific IgG antibodies followed by protein A/G-coated gold nanoparticle (PAG) of different sizes (PAG 10, PAG 15 nm in diameter) were used for labeling of ultrathin sections of \textit{P. aeruginosa} PA14. The anti-DnaK antibodies revealed the predominant localization of DnaK at the cell periphery and in the extracytoplasmic region (Figs. 4B - D). Almost no labeling of \textit{P. aeruginosa} PA14 was observed using PAG nanoparticles alone in the absence of specific antibody (Fig. 4A). Then we performed a second control, which is depicted in Fig.4 B, where after labeling DnaK with specific antibodies possibly free binding sites on the first bound antibody were blocked by incubation with protein A alone. In Fig. 4B only scarcely single PAG 10 could be detected (arrow in Fig.4
B) demonstrating the validation of the performed co-localization studies. This method detects proteins localizing less than 25 nm from each other, since each of the two applied antibodies can maximally span a distance of approximately 12 nm due to the hinge in the Fab region. Double labeling with anti-DnaK and anti-FliC as well as anti-NirS antibodies first revealed that both FliC and NirS proteins were much less abundant than DnaK (Figs. 4C and D). Performing the reversed experiments, e.g. first incubating with anti-NirS and subsequently with anti-DnaK antibodies as well as for the other possible pairings (FliC and DnaK), we observed the same results as for the original experiment. However, when they were detected, they were mostly co-localized with DnaK, providing ultrastructural support for direct DnaK:NirS and DnaK:FliC interaction in the bacterial periplasm.

The NirS is directly interacting via its cytochrome c domain with FliC. In order to obtain further information on the protein-protein interactions in the detected NirS-DnaK-FliC triad, we repeated the bait:prey binding studies as shown in Fig. 1, in \( fliC^- \) (Fig. 5A) and \( nirS^- \) (Fig. 5B) mutant backgrounds. This approach tested for sole DnaK-FliC and DnaK-NirS interaction. As can be seen in Fig. 5, FliC is not required for interaction of NirS with DnaK (Fig. 5A) nor is NirS required for FliC interaction with DnaK (Fig. 5B). Obviously, both NirS and FliC are directly linked to DnaK. To specify the interaction of FliC and NirS we analyzed the complete peptide composition from the samples obtained from the tagged FliC and NirS. Isolated protein complexes were subjected to trypsinisation and peptide analysis by LC-MS/MS (Fig. A3). In these experiments NirS:FliC crosslinked peptides were identified showing a direct interaction. The NirS:FliC peptides from the crosslinked complex were
AAEQYQGAASAVDPTHVVR, CAGCHGVLRK and GQQYLEALITYGTPLGMPNWGSSGELSK, from NirS, and NQVLQQAGTAIAQANQLPQAVALSLR and LGITASINDK, from FliC. Since interaction surfaces of the complex are potentially contained within these peptides, we wanted to map them onto available structure models. As can be seen in Fig. 5C, which shows the NirS peptides that cross link to FliC highlighted in the NirS structure model (obtained from the PDB database (pdb accession code 1NIR (32)), these peptides are located in the cytochrome c domain of NirS (2, 32). Unfortunately, the entire structure, especially the detected peptide containing regions, of the *P. aeruginosa* FliC was not available. Consequently it was not possible to deduce a model of the possible NirS-FliC docking surfaces.

**DISCUSSION**

In this study a novel triple complex consisting of the denitrification enzyme nitrite reductase (NirS), the flagellar filament protein FliC and the chaperone DnaK was identified in the periplasm of *P. aeruginosa* PA14. The current understanding of FliC function localizes the protein in the flagellum outside the cell, while the ATP-dependent DnaK chaperone is considered to be cytoplasmic. However, *E. coli* FliC and several other FliC proteins including *P. aeruginosa* FliC were detected in the inner membrane fraction and the periplasm during various proteomic analyses (38-43). In multiple approaches the FliC protein was detected together with NirS (41) or even with NirS and DnaK (43). Similarly, DnaK was found in the cell envelope of *Aggregatibacter actinomycetemcomitans* (44), *Vibrio cholerae* (14) and crosslinked to *E. coli*
respiratory formate dehydrogenase during mapping of cell envelope and periplasmic protein interaction partners (45). Even a first mechanism for the transport of DnaK into the periplasm of *E. coli* during osmo stress was described (46, 47). In this case the mechano-sensitive channel protein McsL was involved in the export of DnaK. However, other examples for the involvement of DnaK in the export of periplasmic and outer membrane proteins even in the presence of a defect SEC system pointed towards alternative protein export systems involving DnaK (48). All these observations point toward a periplasmic function of FliC and DnaK. Such periplasmic FliC function was described in the context of the *E. coli* LeoA protein activity during enterotoxin secretion (49). Deletion of *leoA*, led to reduced toxin export, accumulation of FliC in the periplasm and a non-motile phenotype (49). Moreover, FliC function in adhesion and virulence has been described in detail before (50, 51). In this context the existence of various differentially N-glycosylated forms of FliC offer the basis for multiple cellular functions in various cellular compartments (52). Species specific differences in FliC function, indicated by the failure to exchange FliC proteins between for example *E. coli* and *P. aeruginosa*, point toward additional FliC function in these bacteria (53). In contrast FliD can be exchanged without further problems between a variety of bacteria (53). Finally, the detected direct interaction of the cytochrome c domain of NirS with both the proposed long N- and C-terminal helices of FliC might give insights into the structural interconnection of denitrification and motility (54). Overall, we assume that the periplasmic compartment represents a highly ordered and structured cellular organelle in Gram-negative bacteria. Most likely, the membrane associated anaerobic energy generation machinery of denitrification forms a
megacomplex as observed for the electron transport chain in mammalian, yeast and plant mitochondria (55-61). Therefore, since several protein-protein interactions of the involved proteins have already been shown, one can assume the presence of a large denitrification megacomplex also in bacteria. The dynamic, but mostly stable complex of denitrification provides an ideal assembly and mobility platform for the multiple other periplasmic proteins involved in processes like ATP generation, disulfide bond formation, protein and metabolite transport, communication to the outer membrane, environmental signal perception and transduction. One of the major challenges of motility is the trans cytoplasmic, periplasmic and outside formation of the flagellum. Here, highly ordered molecular transport and assembly processes are required. Obviously, the already determined multiple protein-protein contact-forming nitrite reductase most likely serves as a major driving force (25). The NirS-FliC interaction would attach FliC to the proposed megacomplex and consequently stabilize the overall flagellum assembly process. The periplasmic NirS is dedicated via the lipoprotein NirF to the outer membrane, opening the flagellum assembly pathway towards extracellular localization. Association with DnaK, usually stabilizing partly denatured, not completely soluble and instable proteins, might provide the opportunity to stabilize involved, structurally instable forms of FliC prior to cellular export and assembly. Therefore, DnaK might serve as FliC stabilizer and NirS as guide from the cytoplasmic to the outer membrane during the process of flagellum assembly. Future investigation will determine the exact molecular mechanism underlying the NirS-DnaK-FliC triad function.
ACKNOWLEDGMENTS

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43. **Casabona MG, Vandebrouck Y, Attree I, Coute Y.** 2013. Proteomic characterization of *Pseudomonas aeruginosa* PAO1 inner membrane. Proteomics **13**:2419-2423.


47. **Berrier C, Garrigues A, Richarme G, Ghazi A.** 2000. Elongation factor Tu and DnaK are transferred from the cytoplasm to the periplasm of


Table 1. Bacterial strains used in this study.

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a) Shows significant characteristic of the strain. b) Shows the source from which the strain was obtained.
FIGURE LEGENDS

**Fig. 1.** Protein Interaction partners of *P. aeruginosa* NirS and FliC. SDS-PAGE analysis of periplasmic proteins co-purifying with affinity-purified bait proteins NirS (A) and FliC (B). Protein extracts from *P. aeruginosa* without bait protein chromatographed through same affinity material served as negative control (C). +CL and -CL indicate whether or not the cell samples were treated with cross linker prior to protein isolation. Proteins obtained from crosslinked samples were heated to 95°C in SDS-sample buffer to reverse crosslinking prior to loading the gel. The identity of the proteins indicated by arrows was determined via their excision from the gels. The proteins were eluted and tryptic digests were analyzed by LC-MS/MS, as described in Materials and Methods. WB (inset) indicates western-blot confirmation of the identity of the bait protein. The relative molecular masses (Mr) x 1000 are indicated.

**Fig. 2.** Swimming motility assays of the *P. aeruginosa* wild-type (WT) and strains carrying inactivated genes for various enzymes of denitrification. Soft agar plates containing arginine as energy source were point inoculated with overnight cultures of the strains to be tested, incubated anaerobically at 37°C for 5 days, as described in Materials and Methods. (A). B) The diameters of the halos of bacterial growth, representing the distance migrated by the strain via swimming, were measured. The plots in B show the mean halo-diameters of triplicate measurements, and their standard deviations.

**Fig. 3.** Flagella morphology in wild-type and nirS mutant visualized by immunofluorescence microscopy. For visualization of extracellular FliC protein, samples of bacteria cultured anaerobically in LB containing arginine
were labeled with primary rabbit anti-FliC antibody followed by secondary Alexa-Fluor 488 conjugated goat anti-rabbit antibody (green), and the DNA was labelled with DAPI (blue). Arrow heads point to nonfunctional flagella in the NirS mutant strain. Fluorescence images were taken and processed as described in Materials and Methods. Scale bars: 10 µm.

Fig. 4. Cellular localization and co-localization of DnaK, FliC and NirS in anaerobically-grown, denitrifying cells of *P. aeruginosa* PA14. Cells were prepared for transmission electron microscopy, treated with specific antibody and Protein A/G conjugated with 10 or 15nm diameter gold nanoparticles (PAG 10, PAG 15, respectively), counterstained, and examined in a TEM910 transmission electron microscope, as described in Materials and Methods. A: antibody-negative control with PAG 15; B: anti-DnaK antibodies with PAG 15, followed by protein A blocking, then PAG 10; C: anti-DnaK antibodies with PAG 15, followed by protein A blocking, then anti-FliC antibodies with PAG 10; D: anti-DnaK antibodies with PAG 15, followed by protein A blocking, then anti-NirS antibodies with PAG 10. DnaK is seen to be distributed mostly in the extracytoplasmic region; several co-complexes between DnaK and FliC, and DnaK and NirS are observed and indicated by arrows. Scale bars are 500 nm for A, B and 200 nm for C, D.

Fig. 5. Structural basis of NirS-FliC-DnaK interactions. A) NirS co-purification with DnaK as bait in the PA14 *fliC*/*nirS*+ strain and B) FliC co-purification in the *P. aeruginosa* nirS*/fliC*+ strain. C) Structural model of dimeric *P. aeruginosa* NirS showing the peptides hypothesized to participate in the interaction with FliC. These peptides were determined as outlined in Fig. A3. NirS structure displayed as cartoon model with transparent surface as inferred
From pdb 1NIR (32). Stick models indicate the heme c (red) and heme d molecules (yellow). Interacting peptides are shown in light gray (AAEQYQGAASAVDPTHVVR), cyan (CAGCHGVLRK) and light blue (GQQYLEALITYGTPLGMPNWGSSGELSK).
Figure 1

A

\[ P. aeruginosa \quad \text{nir}^S/\text{nir}^+ \]

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\[ \text{DnaK} \quad \text{NirS} \quad \text{FliC} \]

B

\[ P. aeruginosa \quad \text{fli}^C/\text{fli}^+ \]

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\[ \text{DnaK} \quad \text{NirS} \quad \text{FliC} \]

C

\[ P. aeruginosa \quad \text{pAS40} \]

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\[ \text{FliC} \]

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Figure 2

A

WT  nirS  fliC  nirS/nirS\textsuperscript{*}  fliC/fliC\textsuperscript{*}  norB  narH  nosZ  nirF

B

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Figure 3

WT

nirS

DIC

anti-FliC

anti-FliC DAPI

merge