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## SUPPLEMENTARY DATA

# Maturation of the cytochrome $cd_1$ nitrite reductase NirS from *Pseudomonas aeruginosa* requires transient interactions between the three proteins NirS, NirN and NirF

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## MATERIALS AND METHODS

### *In vivo* protein cross-linking

The *in vivo* protein cross-linking was performed as described previously with minor modifications. *P. aeruginosa* PAO1 strains were grown for 8 h at 37°C under anaerobic conditions in LB (Luria–Bertani) medium supplemented with 50 mM NaNO<sub>3</sub>. The cultures were then supplemented with 0.125% of formaldehyde solution (37%) and incubated for 20 min at 37°C on an orbital shaker at 160 rpm. Cross-linking was stopped by addition of 125 mM (final concentration) glycine solution and incubation for 5 min at 37°C and 160 rpm.

### Western blotting

For the detection of NirS, NirN or STREP-tagged NirF variants, samples were supplemented with SDS-sample buffer containing 2-mercaptoethanol, heated at 95°C for 10 min and the proteins were separated on 12% denaturing polyacrylamide gels. Afterwards, the proteins were blotted onto polyvinylidene fluoride membranes. Staining of the proteins was performed with the monoclonal antibodies 1A11 ( $\alpha$ NirS) and 2C11 ( $\alpha$ NirN) and alkaline phosphatase attached to a second-

ary antibody. NirF-OneSTREP was directly probed with Strep-Tactin AP conjugate after masking biotinylated proteins with avidin.

### Preparation of immuno-affinity resin

Protein A- and protein G-resin was mixed in a 1:1 ratio to give a 1 ml column volume and equilibrated with binding buffer according to the manufacturer's manual (GenScript). 1 mg of monoclonal antibodies ( $\alpha$ NirS-mix) or polyclonal antibodies ( $\alpha$ NirN) were bound to the column and washed with 50 column volumes (CV) of binding buffer at a flow rate of 1.3 ml/min. Afterwards, the immuno-affinity resin was stored in binding buffer supplemented with 0.02% of NaN<sub>3</sub> or directly washed with 30 CV of 0.2 M triethanolamine, pH 8.2. The cross-linking of the antibodies with the immuno-affinity resin was performed with 20 CV of 70 mM dimethyl pimelimidate in 0.2 M triethanolamine, pH 8.2, for 45 min at room temperature. The resin was then collected by soft centrifugation and separation from the supernatant. The cross-linking was stopped by resuspending the resin in 25 CV of 70 mM ethanolamine, pH 8.2, and incubation for 5 min at room temperature. The resin was washed with 50 CV of binding buffer and stored for a short time in binding buffer containing 0.02% NaN<sub>3</sub>.

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**Table S1 Oligonucleotides and primers**

Restriction sites are underlined

Number	Sequence (5'→3')	Use
1	GACCATGGGTAAGGACGACATGAAAG	Amplification of <i>nirS</i> fw. without leader sequence ( <i>NcoI</i> )
2	GTAAGCTTCAGTACACGTGCTGGG	Amplification of <i>nirS</i> rev. ( <i>HindIII</i> )
3	CATGCCATGGGCGAAGCGCCG	Amplification of <i>nirN</i> fw. without leader sequence ( <i>NcoI</i> )
4	CGGGATCCTCAGTGCGAGGTTT	Amplification of <i>nirN</i> rev. ( <i>BamHI</i> )
5	CATCGGATCCAGGAGAGATCGCC	Amplification of <i>nirF</i> fw. with <i>rbs</i> and leadersequence ( <i>BamHI</i> ) and <i>nirFOneStrEP</i> fw. ( <i>BamHI</i> )
6	CATCACTAGTGAGTCCGATGTGCTGGG	Amplification of <i>nirF</i> rev. without stop codon ( <i>SpeI</i> )
7	CATCGCATGCTCACTACTTCTCGAACTG	Amplification of <i>nirFOneStrEP</i> rev. ( <i>SphI</i> )
8	GTGCGGCCGCAAGCTTCTAGAGTCCGATGTGCTGG	QuikChange <i>nirF</i> fw. incorporate stop codon
9	CCAGCACATCGGACTCTAGAAGCTTGCGGCCGCAC	QuikChange <i>nirF</i> rev. incorporate stop codon
10	GCCGCCGGAGCCGCCCTACTTCTCGAACTGGGG	QuikChange <i>StreptII</i> fw. shorten <i>StrEPOne</i> Tag
11	CCCCAGTTCGAGAAGTAGGGCGGCTCCGGCGGC	QuikChange <i>StreptII</i> rev. shorten <i>StrEPOne</i> Tag

**Table S2 Periplasmic (P), membrane proteins (IM/OM) and outer membrane vesicle proteins (OMV) from *P. aeruginosa* PA01**

Corresponds to Figure 4(A) of the main text. Locations according to www.pseudomonas.com or SignalP3.0 Server. Trypsin-digested. Peptide Mass Tolerance,  $\pm 20$  p.p.m. Fragment Mass Tolerance,  $\pm 0.3$  Da. Significance Threshold  $P < 0.05$ . First 10 Results or Score above 45.

(a) Band, NosZ; band mass value, about 70 kDa

gi	Gene	Protein	Mass	Score	Sequences	Location
15595716	PA0519	NirS	62614	536	19	P
15598588	PA3392	NosZ	70615	150	7	P
15597002	PA1805	PpiD	68699	148	7	IM
15599791	PA4595	ABC-Transporter ATP Binding	61283	106	4	P
15597007	PA1810	ABC-Transporter component	70429	96	4	P
15599939	PA4745	NusA	54626	95	4	P
15596780	PA1583	SdhA	63492	76	2	P
15595785	PA0588	Hypothetical	73676	76	3	OMVesicle
15595715	PA0518	NirM	10960	66	1	P
15600230	PA5037	Hypothetical	57412	61	2	IM

(b) Band, NirS; band mass value about 60 kDa.

gi	Gene	Protein	Mass	Score	Sequences	Location
15595716	PA0519	NirS	62614	7843	35	P
15599581	PA4358	GroEL	57050	685	15	P/OMVes.
15599698	PA4502	ABC-Transporter component	58575	132	4	P
15599687	PA4491	Hypothetical	64175	78	3	OMVesicle
15596997	PA1800	Tig	48552	66	2	P
15596580	PA1383	Hypothetical	63104	65	2	P
15597001	PA1804	HupB	9081	57	2	OMVesicle
15598994	PA3799	EngA	54972	57	3	IM
15598588	PA3392	NosZ	70615	54	2	P
15600244	PA5051	ArgS	65158	53	2	P

(c) Band, NirN; band mass value, about 50 kDa

gi	Gene	Protein	Mass	Score	Sequences	Location
15595716	PA0519	NirS	62614	411	10	P
15596997	PA1800	Tig	48552	224	9	P
15597001	PA1804	HupB	9081	218	4	OMVesicle
15598941	PA3746	Ffh	49328	161	3	IM
15595706	PA0509	NirN	53945	99	2	P
15600432	PA5239	Rho	47154	93	4	P
15596352	PA1155	NrdB	47356	76	3	P
15599026	PA3831	PepA	52299	61	2	OMVesicle
15596202	PA1005	Hypothetical	52961	60	2	P
15598197	PA3001	Glycerald.-3-phosph. dehyd.	50080	60	2	P

(d) Band, NirF; band mass value, about 40 kDa

gi	Gene	Protein	Mass	Score	Sequences	Location
15595716	PA0519	NirS	62614	863	17	P
15595791	PA0594	SurA	48443	220	9	P
15596169	PA0972	TolB	47722	219	7	P
15600135	PA4942	HflK	44018	203	4	OMVesicle
15596271	PA1074	BraC	39744	192	5	P
15598813	PA3617	RecA	36856	185	4	C/P/OMV
15600451	PA5258	Hypothetical	40767	170	6	IM
15595713	PA0516	NirF	43311	170	6	P
15596785	PA1588	SucC	41517	111	4	P
15598355	PA3159	WbpA	48125	106	3	P/OMV

**Table S3 Periplasmic (P), membrane proteins (IM/OM) and outer membrane vesicle proteins (OMV) from *P. aeruginosa* PA01**

Corresponds to Figure 4(B) of the main text. Locations according to [www.pseudomonas.com](http://www.pseudomonas.com) or SignalP3.0 Server. Trypsin-digested. Peptide Mass Tolerance,  $\pm 20$  p.p.m. Fragment Mass Tolerance,  $\pm 0.3$  Da. Significance Threshold  $P < 0.05$ . First 10 Results or Score above 45.

(a) Band, NirS; band mass value, about 60 kDa

gi	Gene	Protein	Mass	Score	Sequences	Location
15599581	PA4358	GroEL	57050	731	15	P/OMVes.
15595716	PA0519	NirS	62614	522	12	P
15599525	PA4329	PykA	52220	339	8	P
15598172	PA2976	Rne	117395	276	8	OMVes.
15599069	PA3874	NarH	58068	264	7	OMVes.
15600271	PA5078	Glucon biosynth. Protein G	59439	212	7	P
15596997	PA1800	Tig	48552	151	4	P
15600749	PA5556	AtpA	55359	136	4	P
15597035	PA1838	CysI	62094	122	3	P
15598358	PA3162	RpsA	61832	111	2	P/OMVes.

(b) Band, NirN; band mass value, about 50 kDa

gi	Gene	Protein	Mass	Score	Sequences	Location
15600432	PA5239	Rho	47040	525	14	P/IM
15595706	PA0509	NirN	53945	383	9	P
15596997	PA1800	Tig	48552	352	10	P
15596352	PA1155	NrdB	47356	213	4	P
15599581	PA4358	GroEL	57050	201	4	P/OMVes.
15598197	PA3001	Glyceraldehy.-3-phosph DH	50080	199	5	P/OMVes.
15596291	PA1094	FliD	49420	196	6	P/OMVes.
15600747	PA5554	AtpD	49469	188	5	P/OMVes.
15598172	PA2976	Rne	117395	170	5	OMVes.
15600189	PA4669	RfaE	50318	162	5	P

(c) Band, NirF; band mass value, about 38 kDa

gi	Gene	Protein	Mass	Score	Sequences	Location
15596534	PA1337	AnsB	38620	195	7	P
15600425	PA5232	Hypothetical	38549	135	3	OMVesicle
15598363	PA3167	SerC	39924	120	2	P
15597638	PA2442	GcvT2	39857	85	3	P
15595497	PA0300	SpuD	40604	83	2	P
15599581	PA4358	GroEL	57050	72	2	P/OMVes.
15597936	PA2740	PheS	38039	70	2	P
15600035	PA4842	Hypothetical	39964	65	1	OMVesicle
15598734	PA3538	ABC-Transporter component	39166	61	2	IM
15595713	PA0516	NirF	43311	60	2	P

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