The anti-activator ExsD forms a 1:1 complex with ExsA to inhibit transcription of type III secretion operons

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SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Plasmid constructions for heterologous system. For the construction of the reporter gene plasmid, the promoter of *exsCEBA* operon (*pC*) was amplified by PCR using primers PSE26 and PSE27, cloned into pUC18 cut by *Sma*I and sequenced. The 0.37-kb fragment was released by *Bam*HI and cloned into mini CTX-*lacZ* leading to plasmid pCTX-pC^+lacZ. The pRK-pC^+lacZ plasmid was constructed by cloning the 4.5-kb *Xba*I-*Hin*dIII fragment from pCTX-pC^+lacZ into pSE102 (see Table S1).

To construct the *exsA* expression vector, the gene was first amplified using primers PSE14 and PSE15, cloned into pUC18 cut by *Sma*I, and sequenced. A fragment of 0.85-kb was excised using *Nde*I-*Bam*HI and cloned into pET-15b, yielding pET15b-HExsA. This plasmid was hydrolyzed with *Nde*I and *Eco*RV, and the fragment of 0.98-kb was inserted into pIApC which was previously cut by *Hin*dIII, then treated with the Klenow fragment of DNA polymerase I and finally cut by *Nde*I. The resulting plasmid, pPES29, was further cut with *Pvu*I, treated with T4 DNA polymerase and then hydrolyzed by *Xba*I to obtain a 1.2-kb fragment: the fragment was put under the control of the pX_2 promoter in pIApX_2 cut by *Hin*dIII, treated with Klenow, then *Xba*I. The pX_2ExsA fragment (2.17-kb fragment obtained by *Pvu*II hydrolysis of pIApX2-ExsA) was cloned into pRSFDuet-1 cut by *Pvu*II, that generated the final pRSF-pX_2ExsA plasmid.

To construct the *exsD* expression vector, the gene was amplified using primers PSE20 and PSE21, cloned onto pUC18 cut by *Sma*I, then sequenced. The gene was hydrolyzed by *Nco*I and *Bam*HI, and the resulting 0.84-kb fragment cloned into the same sites in pET-15b, leading to pPES18. The pIApX_2ExsD plasmid was constructed by replacing the 0.76-bp *gfp* gene of plasmid pIApX_2 (cut with *Hin*dIII, treated by Klenow then hydrolyzed with *Xba*I) by the 1.07-kb fragment of pPES18 obtained by *Eco*RV and *Xba*I hydrolysis.

Construction of overexpression plasmids. The 0.84-kb DNA fragment encoding full length ExsA was PCR amplified with PSE14 and PSE22 primers. The PCR product was cloned into pUC18 cut with *Sma*I and sequenced. The *Nde*I-*Hin*dIII fragments was then excised from the plasmid and cloned into pET-22b hydrolysed by *Nde*I/*Hin*dIII. The resulting pET22b-ExsAH plasmid and the already described pET15b-HExsA plasmid (see above) allow, respectively, the overproduction of the ExsA-His_6 and His_6-ExsA fusion proteins. Two fragments encoding either the N-terminal domain of ExsA ($ExsA_{1\text{-}168}$) or the C-terminal domain of ExsA ($ExsA_{166\text{-}278}$) were amplified by PCR using the primers PSE14/PSE43 or PSE34/PSE36, cloned into pCR-Blunt II-TOPO and sequenced. The 0.51-kb and 0.35-kb *Nde*I-*Bam*HI fragments were then cloned into pET-15b. The resulting pET15b-HNter and pET15b-HCter plasmids were used for the overproduction of the His_6-Nter ExsA and His_6-Cter ExsA fusion proteins, respectively. The *exsD* gene, amplified using primers PSE20/PSE21 and cloned into pUC18, was further cloned into pACYCDuet-1 plasmid as a *Nco*I/*Bam*HI fragment. The resulting pACYC-ExsD plasmid was used to overproduce the ExsD protein.

TABLE S1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli strains		Invitrogen
TOP10	Chemically competent cells	Invitrogen
BL21 Star (DE3)	F^- *ompT* $hsdS_B$ (r_B^- m_B^-) *gal dcm rne131*(DE3)	
Plasmids		
pUC18	Ap^r; Cloning vector	(1)
pCR-Blunt II-TOPO	Kn^r; Cloning vector	Invitrogen
pET-15b	Ap^r; Expression vector	Novagen
pET-22b	Ap^r; Expression vector	Novagen
pRSFDuet-1	Kn^r; Expression vector	Novagen
pACYCDuet	Cm^r; Expression vector	Novagen
pSE102	Tc^r; broad-host-range pRK290-derived vector	(2)
pRK-*pC*$^+$*lacZ*	Tc^r; pSE102 containing the *pC*$^+$*-lacZ* fusion	*This study*
pIApX_2	Ap^r; pUCP20-derived plasmid containing the *pX*$_2$-*gfp*mut3 fusion[b]	I. Attree, unpublished
pIApX_2ExsD	Ap^r; pIApX_2 plasmid bearing the *pX*$_2$*exsD* fusion	*This study*
pRSF-pX_2ExsA	Kn^r; pRSFDuet-1 containing the p*X*$_2$*-exsA* fusion	*This study*
pET15b-HExsA	Ap^r; Expression vector of *his*$_6$*-exsA*	*This study*
pET22b-ExsAH	Ap^r; Expression vector of *exsA-his*$_6$	*This study*
pET15b-HNter	Ap^r; Expression vector of *his*$_6$*Nter-exsA*	*This study*
pET15b-HCter	Ap^r; Expression vector of *his*$_6$*Cter-exsA*	*This study*
pET15b-PcrV	Ap^r; Expression vector of *his*$_6$*pcrV*	(3)
pACYC-ExsD	Ap^r; Expression vector of *exsD*	*This study*

[a] Ap^r, Cm^r, Kn^r, Tcr^r indicate resistance to ampicillin, chloramphenicol, gentamycin, kanamycin, and tetracycline, respectively.

[b] pX_2: T3SS-independent and constitutive promoter isolated from the genome of the *P. aeruginosa* mucoid cystic fibrosis isolate CHA

References

1. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) *Gene* **33,** 103-119
2. Elsen, S., Duché, O., and Colbeau, A. (2003) *J. Bacteriol.* **185,** 7111-7119
3. Schoehn, G., Di Guilmi, A. M., Lemaire, D., Attree, I., Weissenhorn, W., and Dessen A. (2003). *EMBO J*. **22,** 4957-4967.

TABLE S2. Oligonucleotides used in this study

Primer[a]	DNA sequence[b]
Cloning	
PSE14	5' CATATGCAAGGAGCCAAATCTCTTG 3' (*Nde*I)
PSE15	5' ACGTCAGTTATTTTTAGCCCGGC 3'
PSE20	5' CCATGGAGCAGGAAGACGATAAG 3' (*Nco*I)
PSE21	5' GCCGCTCAGCTCTGCCAGTAG 3'
PSE22	5' AAGCTTGTTATTTTTAGCCCGGCATTC 3' (*Hin*dIII)
PSE26	5' GGATCCAGGTGCGCCAGGGCG 3' (*Bam*HI)
PSE27	5' GGATCCTGCGAACTCGGCAAGCAG 3' (*Bam*HI)
PSE34	5' CATATGAGCAACCGGCATGTCGAG 3'(*Nde*I)
PSE36	5' GGATCCACGTCAGTTATTTTTAGCCCG 3' (*Bam*HI)
PSE43	5' GGATCCTCACCGGTTGCTCAGTTGCCG 3'(*Bam*HI)
EMSA	
5'dR Biot-pC EMSA F	5'GCGATGTGGCTTTTTTCTTAAAAGAAAAGTCTCTCAGTGACA AAAGCGATGCATAGCCCG 3'
5'dR Biot-pC EMSA R	5'CGGGCTATGCATCGCTTTTGTCACTGAGAGACTTTTCTTTTA AGAAAAAAGCCACATCGC 3'
SEC	
pC EMSA F	5'GCGATGTGGCTTTTTTCTTAAAAGAAAAGTCTCTCAGTGACA AAAGCGATGCATAGCCCG 3'
pC EMSA R	5'CGGGCTATGCATCGCTTTTGTCACTGAGAGACTTTTCTTTTA AGAAAAAAGCCACATCGC 3'

[a]The primers were either used during the cloning steps, the Electrophoretic Mobility Shift Assays (EMSA), or the size exclusion chromatography (SEC) study

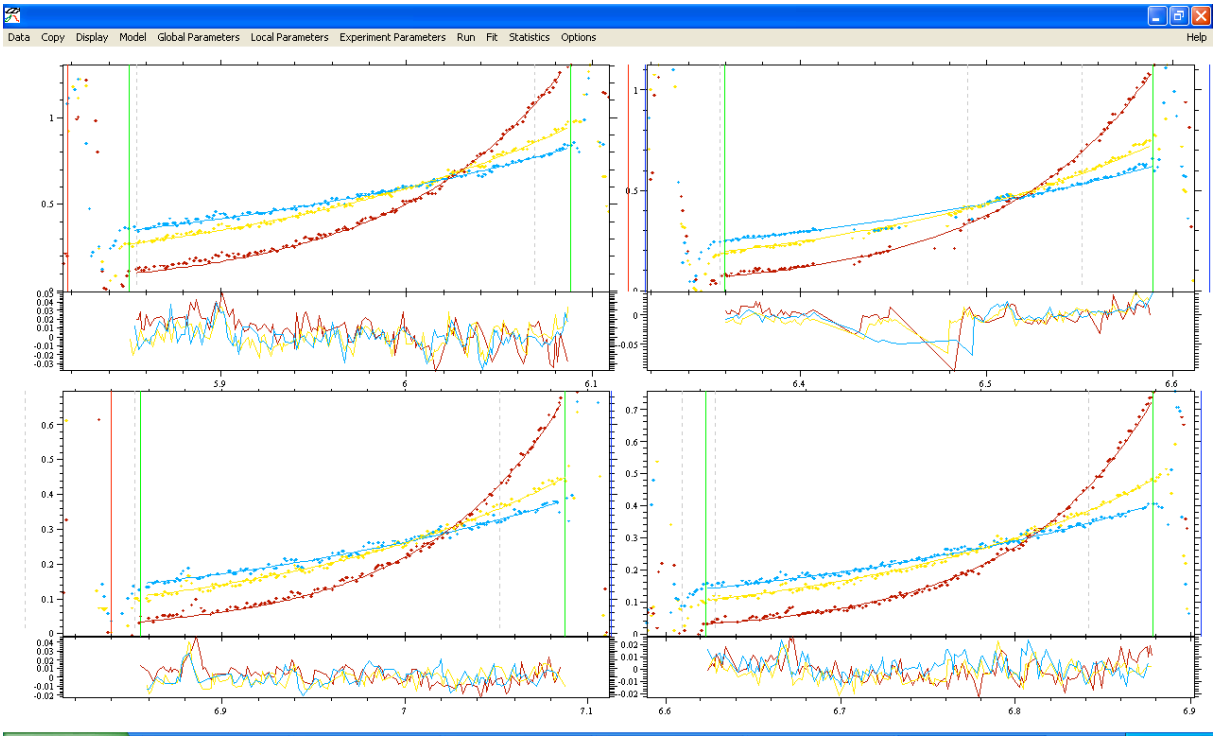
[b]Restriction sites incorporated into primers are underlined and indicated in brackets

Table S3. Molar masses, partial specific volumes and extinction coefficients of $ExsA_H$, $_HExsA$, ExsD and complexes.

	A_H	$_HA$	D	A_H/D	$A_H/2D$	$2A_H/D$	$_HA/D$	$_HA/2D$	$2_HA/D$
M (Da)	33082	33798	31372	64454	95826	97536	65170	96542	98968
$\bar{v}$ 20°C (ml g^{-1})	0.740	0.737	0.731	0.735	0.734	0.737	0.734	0.733	0.735
ε_{280} ($cm^{-1}M^{-1}$)	34360	34360	73890	108250	182140	142610	108250	182140	142610
$E_{280,0.1\%}$ ($cm^{-1}mg^{-1}ml$)	1.04	1.02	2.36	1.68	1.90	1.46	1.66	1.89	1.44

M: molar mass; $\bar{v}$: partial specific volume; ε_{280} : molar extinction coefficient at 280 nm; $E_{280,0.1\%}$: absorbance at 280 nm for 1 mg/ml and 1 cm optical pathlength of A_H: $ExsA_H$; $_HA$: $_HExsA$; D: ExsD and complexes. These values were calculated from the aminoacid composition using the program Sednterp (www.jphilo.mailway.com).

Figure S1. Equilibrium sedimentation of $ExsA_H$/ExsD samples at 4°C.



Sedimentation equilibrium profiles were obtained at 8,300 rpm (blue), 10,000 rpm (yellow) and 14,000 rpm (red) for solutions of complex at 0.33, 0.23, 0.14 and 0.58 mg/ml (top left, top right, bottom left and bottom right panels respectively). The first three samples were measured in 6-channel of 1.2 cm optical pathlength and the latter in a 2-channel centrepiece of 0.3 cm optical pathlength. Each panel shows in the top sub-panel the superimposition of the experimental (points) and fitted (curves) data, and in the bottom sub-panel the superimposition of the residuals (differences between the experimental and fitted data). The analysis was made using Sedphat software (www.analyticalultracentrifugation.com) considering the raw data, with one same species in all samples, with mass conservation in each sample, leading to a (common) molar mass of 70.2 kDa, calculated with a partial specific volume of 0.73 ml/g and absorbencies of 0.57, 0.40, 0.25 (in the 12 mm optical path-length cells) and 0.25 (in the 3 mm optical path-length cell), with local root mean square deviations of 0.015, 0.016, 0.010 and 0.008 absorbance units (top left, top right, bottom left and bottom right panels respectively).