Supplementary Material for

A global view of *Escherichia coli* Rsd protein and its interactions

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Rsd expression and quantification

E. coli MG1655 cells were grown aerobically overnight (with vigorous shaking) in LB medium at 37 °C, followed by a 100-fold dilution of the overnight culture into fresh LB medium (shaken aerobically at 37 °C). Samples were harvested throughout growth and the number of cells in each harvested sample was calculated using the viable cell count, obtained from plating serial dilutions of selected samples onto LB plates, and comparing it with the corresponding OD_{650} measurement. The doubling time of MG1655 cells, determined from OD_{650} measurements, was calculated to be 36 minutes (1.6 doublings per hour). Lysates, prepared from samples of cell extracts corresponding to 8.10×10^6 cells, were loaded onto an SDS PAGE gel and western blot analysis was performed (Figure S1A). Proteins were electroblotted onto nitrocellulose membranes and probed firstly with rabbit anti-Rsd sera and secondly with horseradish peroxidase-conjugated anti-rabbit antibodies. Blots were developed using chemiluminescent detection reagents, and the signal was visualised by exposure to autoradiography film. To quantify Rsd, Quantity One software (Bio-Rad) and a GeneGenius Bio Imaging system with a CCD camera (Syngene) was used to compare densitometric measurements of band intensities. To generate a calibration curve, samples of purified Rsd of known concentration were run on SDS PAGE, and the signal intensities of each of these standards was fitted to a straight line (Figure S1B). Band intensities were compared against the standards to determine the mass (ng) of Rsd protein in each sample. Hence, the number of molecules of Rsd per cell was calculated for each growth point (Figure S1C). This was repeated three times and an average was taken of the number of molecules in exponential (OD_{650} 0.3) and stationary phase (OD_{650} 3.0-4.0). The full results are shown in Table S1.

Quantification of RNA polymerase subunits

The levels of σ^{70}, σ^{38} and core RNA polymerase subunits were also measured throughout growth in MG1655, using western blot analysis, as for Rsd. To determine the number of molecules of core RNA polymerase, the intracellular levels of the individual α, β and β' subunits were measured. It has been shown that all of the β and β' subunits present in the cell are incorporated into the core RNA polymerase complex within 5 minutes of synthesis, and consequently there is no accumulation of free β or β' subunits or intermediate complexes (Iwakura *et al*., 1974). For this reason, the determination of the intracellular level of β and β' is considered to be a reliable indicator of the total number of core RNA polymerase molecules. The measurement of the amount of α subunit is less reliable as studies have shown that there is some unincorporated α subunit in the cell; however the intracellular level was measured for comparison (Iwakura *et al*., 1974).

MG1655 cells were grown aerobically overnight (with vigorous shaking) in LB medium at 37 °C, followed by a 100-fold dilution of the overnight culture into fresh LB medium (shaken aerobically at 37 °C). Samples were harvested throughout growth, OD_{650} measurements were taken, and cell lysates were prepared. Samples of cellular extracts corresponding to 8.10×10^6 cells were then analysed by SDS PAGE and western blot analysis was performed, probing firstly with anti-σ^{70}, anti-σ^{38}, anti-α, anti-β or anti-β' (all raised in mice) and secondly with a horseradish peroxidase-conjugated anti-mouse antibody. The signal was then quantified using densitometric measurements as described above. For estimation of the intracellular levels of σ^{70} and σ^{38}, samples of known concentrations of each purified subunit were loaded onto the gels. For estimation of the levels of α, β and β', samples of purified holoenzyme ($E\sigma^{70}$) of known concentrations (from Epicentre) were loaded onto the gels. The resulting western blot images are shown in part A of Figures S2-S6. In each experiment, a calibration curve was constructed based on the intensity of the signal from the known standards, and this was used to determine the mass of protein in each unknown sample (part B of Figures S2-S6). The total number of molecules per cell was calculated and the data is shown in part C of Figures S2-S6. Each experiment was repeated three times and an average was taken of the number of molecules in exponential (OD_{650} 0.3) and stationary phase (OD_{650} 3.0-4.0). This data are summarised in Table S1.

The results indicate that σ^{70} is present throughout growth and that its levels remain constant at around 7000 molecules per cell (Figure S2). In contrast, σ^{38} is only detectable during

stationary phase, where it accumulates to around 1300-1600 molecules per cell, approximately 20 % of the level of σ^{70} (Figure S3). The average number of molecules of α present throughout growth was estimated as ~5300-5400 molecules per cell (Figure S4). Since α functions as a dimer, this could give ~2600-2700 molecules of RNA polymerase per cell. A similar estimate was obtained from measurement of the intracellular levels of β and β' (Figures S5 and S6). Assuming that all α, β and β' subunits detected are present as part of the RNA polymerase complex, the average value for the number of molecules of RNA polymerase, obtained from measurement of α, β and β' is ~2500 molecules per cell. RNA polymerase concentrations were taken as $((N \div V) \div A) \div 1 \times 10^{-6}$, where N represents the number of molecules per cell, A represents Avogadro's constant (6×10^{23}) and V represents the volume of an *E. coli* cell which is assumed to be 1×10^{-15} litres (Bremer and Dennis, 1987; Grigorova *et al*., 2006). Calculated concentrations are summarised in Table S2.

Immunoisolation pull-down assays to investigate Rsd-associated proteins

Previous work had shown that Rsd interacts with σ^{70} (Jishage and Ishihama, 1998). To identify any other interacting proteins, immunoisolation was used to purify Rsd together with any associated proteins from MG1655 extracts. This was modelled on a previous study in which RNA polymerase and associated proteins were affinity isolated from *E. coli* O157:H7 (Lee *et al*., 2008). As a starting point, the *rsd* gene of MG1655 was tagged with a segment of DNA encoding the 3xFLAG epitope using the Datsenko and Wanner (2000) method. MG1655 *rsd::3xFLAG* cells were grown aerobically in LB medium at 37 °C, and samples were harvested at OD_{650} 0.8 or 3.7. Cells were disrupted by sonication and Rsd::3xFLAG protein was affinity isolated by incubating cell lysates with magnetic beads coated with anti-FLAG antibody. The beads were then collected using a magnetic rack and the protein complexes were eluted from the beads and analysed by SDS-PAGE. The results (Figure S7) show that the only clearly detectable protein to co-isolate with Rsd is σ^{70}. Figures S8 and S9 show the results of an experiment to quantify the amount of σ^{70} bound to Rsd::3xFLAG in both samples, using quantitative western blot analysis. The experiments shows that, in the sample harvested at OD_{650} 0.8, 1 Rsd molecule in every 15 (~7 % of Rsd) is bound to σ^{70}, whilst, in stationary phase, 1 Rsd molecule in every 4 (25 % of Rsd) is bound to σ^{70}.

Literature cited

R. Bremer and P. Dennis (1987). Modulation of Chemical Composition and Other Parameters of the Cell by Growth Rate. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*. Neidhart, F, C., Ingraham, J, L., Low, K, B., Magasanik, B., Schaechter, M., Umbarger, H, E (ed): ASM press, Washington DC. pp: 1527-1542

K. A. Datsenko and B. L. Wanner (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA.* **97**: 6640-6645

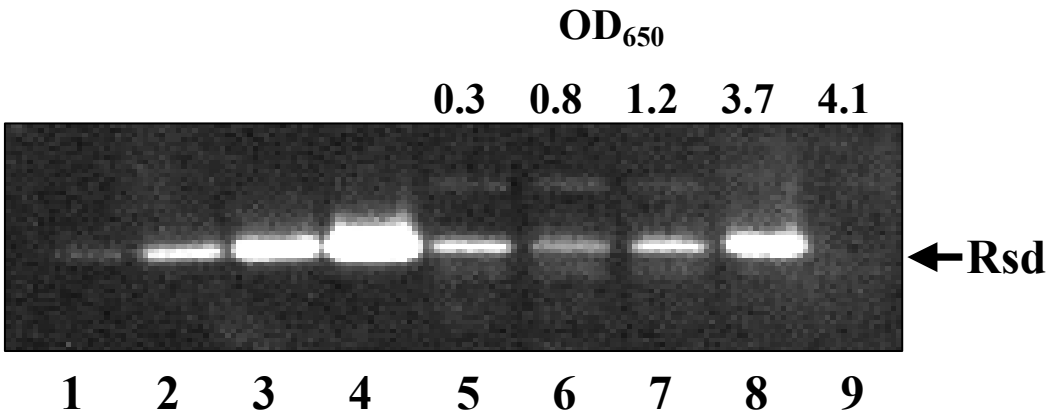
I. L. Grigorova, N. J. Phleger, V. K. Mutalik and C. A. Gross (2006). Insights into transcriptional regulation and σ competition from an equilibrium model of RNA polymerase binding to DNA. *Proc. Natl. Acad. Sci. USA.* **103**: 5332-5337

Y. Iwakura, K. Ito and A. Ishihama (1974). Biosynthesis of RNA polymerase in *E. coli*. I. Control of RNA polymerase content at various growth rates. *Mol. Gen. Genet.* **133**: 1-23

M. Jishage and A. Ishihama (1998). A stationary phase protein in *E coli* with binding activity to the major σ subunit of RNA polymerase. *Proc. Natl. Acad. Sci USA.* **95**: 4953-4958

D. J. Lee, S. J. W. Busby, L. F. Westblade and B. T. Chait (2008). Affinity isolation and I-DIRT mass spectrometric analysis of the *Escherichia coli* 0157:H7 Sakai RNA polymerase complex. *J. Bacteriol*. **190**: 1284-1289

A



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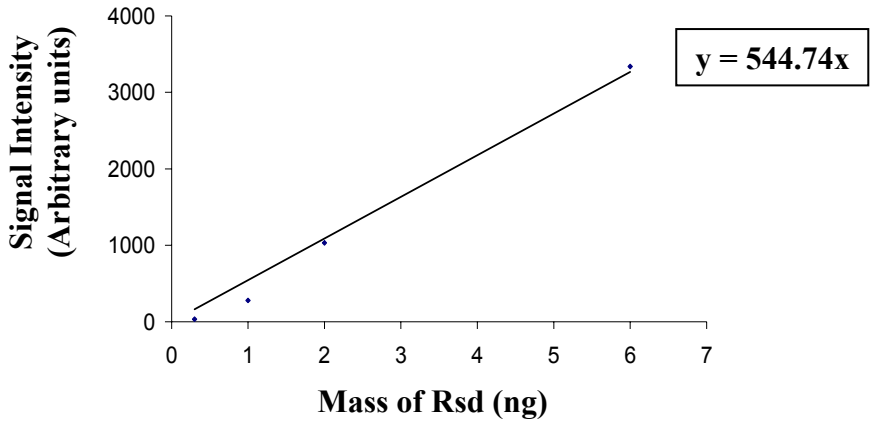

C

Sample	Mass of Rsd (ng)	Number of molecules	Number of cells	Number of molecules per cell
MG1655 OD_{650} **0.3**	0.76	2.54×10^{10}	8.10×10^{6}	**3140**
MG1655 OD_{650} **0.8**	0.70	2.34×10^{10}	8.10×10^{6}	**2892**
MG1655 OD_{650} **1.2**	0.78	2.61×10^{10}	8.10×10^{6}	**3222**
MG1655 OD_{650} **3.7**	1.52	5.09×10^{10}	8.10×10^{6}	**6279**

Figure S1 Quantification of Rsd throughout growth of MG1655

Part A shows a western blot assay using rabbit anti-Rsd sera. Samples of pure Rsd were loaded onto an SDS-PAGE gel in lanes 1-4 (300 pg in lane 1, 1 ng in lane 2, 2 ng in lane 3 and 6 ng in lane 4), and extracts of MG1655 cells grown to different densities were loaded in lanes 5-8. In lanes 5-8 the amount of extract loaded corresponded to 8.10×10^{6} cells. As a control, an extract of MG1655Δ*rsd*::*kan* cells was loaded in lane 9. Part B is a calibration curve relating signal intensity to the mass of Rsd loaded. Part C is a table showing the calculated number of molecules of Rsd per cell for each growth point. The mass of Rsd (ng) (column 2) was estimated from the calibration curve. The number of molecules of Rsd (column 3) was determined by first calculating the number of moles of Rsd (mass (g) ÷ M_r (18,000)) and then multiplying by Avogadro's constant (6.023×10^{23}). The number of molecules of Rsd per cell (column 5) was obtained by dividing the total number of molecules (column 3) by the number of cells in the starting sample (column 4).

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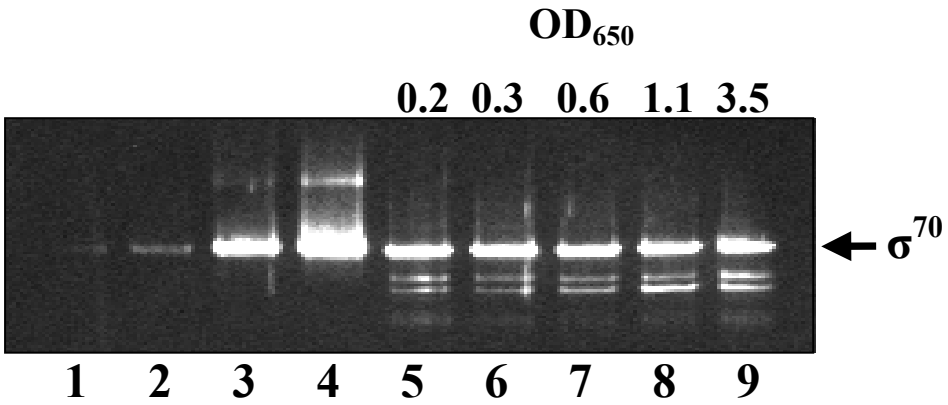

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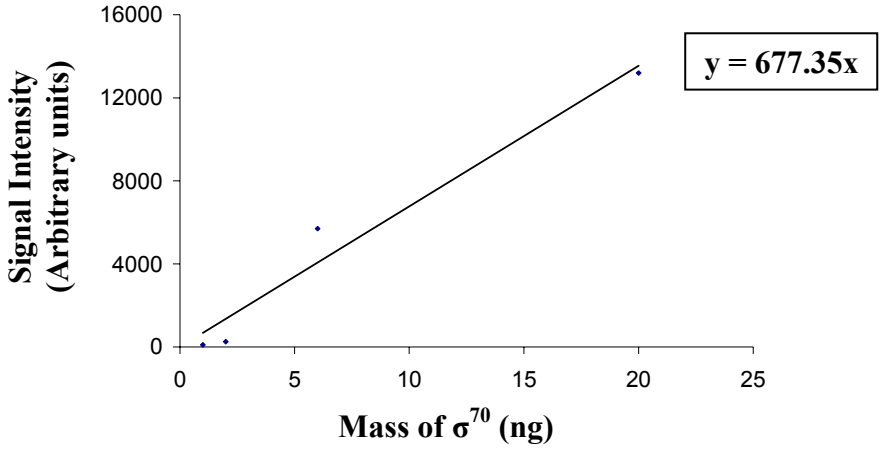


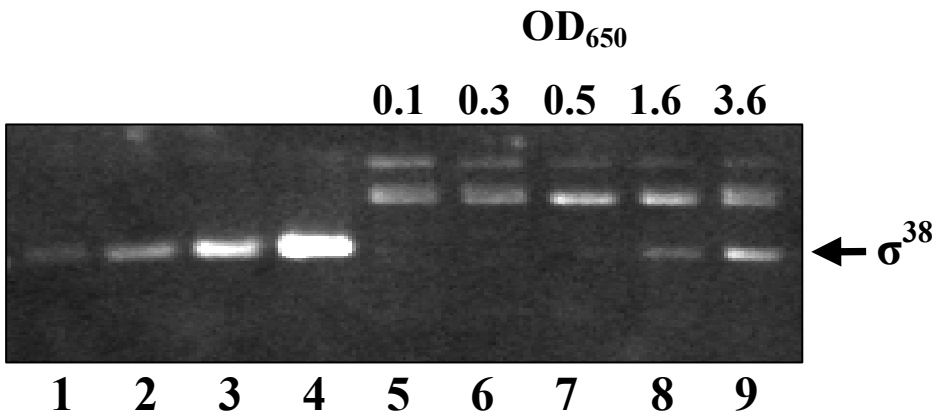
C

Sample	Mass of σ^{70} (ng)	Number of molecules	Number of cells	Number of molecules per cell
MG1655 OD_{650} **0.2**	6.92	5.95×10^{10}	8.10×10^{6}	**7351**
MG1655 OD_{650} **0.3**	6.81	5.86×10^{10}	8.10×10^{6}	**7234**
MG1655 OD_{650} **0.6**	6.90	5.94×10^{10}	8.10×10^{6}	**7330**
MG1655 OD_{650} **1.1**	6.97	6.00×10^{10}	8.10×10^{6}	**7404**
MG1655 OD_{650} **3.5**	6.78	5.83×10^{10}	8.10×10^{6}	**7202**

Figure S2 Quantification of σ^{70} throughout growth of MG1655

Part A of the figure illustrates a western blot assay using mouse anti-σ^{70} sera. Samples of pure σ^{70} were loaded onto an SDS-PAGE gel in lanes 1-4 (1 ng in lane 1, 2 ng in lane 2, 6 ng in lane 3 and 20 ng in lane 4), and extracts of MG1655 cells, grown to different densities, were loaded in lanes 5-9. The amount of cell extract loaded in each lane corresponded to 8.10×10^{6} cells. Part B is a calibration curve relating signal intensity and the mass of σ^{70} loaded. Part C is table in which the number of molecules of σ^{70} per cell for each growth point is calculated as in Figure S1.

A



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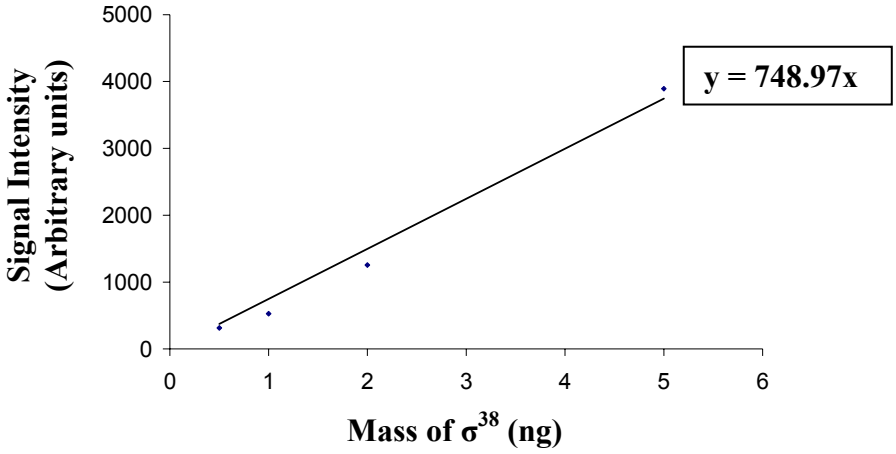


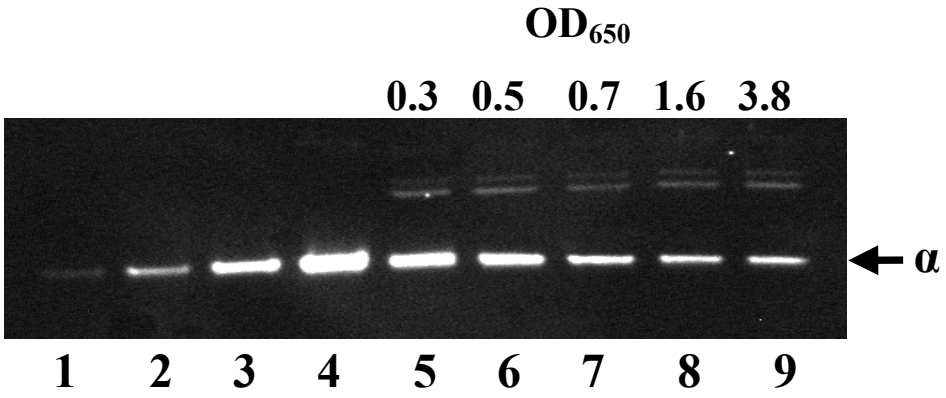
C

Sample	Mass of σ^{38} (ng)	Number of molecules	Number of cells	Number of molecules per cell
MG1655 OD_{650} **0.1**	-	-	8.10×10^6	**Not detectable**
MG1655 OD_{650} **0.3**	-	-	8.10×10^6	**Not detectable**
MG1655 OD_{650} **0.5**	-	-	8.10×10^6	**Not detectable**
MG1655 OD_{650} **1.6**	0.68	1.08×10^9	8.10×10^6	**1331**
MG1655 OD_{650} **3.6**	0.85	1.35×10^{10}	8.10×10^6	**1663**

Figure S3 Quantification of σ^{38} throughout growth of MG1655

Part A of the figure illustrates a western blot assay using mouse anti-σ^{38} sera. Samples of pure σ^{38} were loaded onto an SDS-PAGE gel in lanes 1-4 (500 pg in lane 1, 1 ng in lane 2, 2 ng in lane 3 and 5 ng in lane 4), and cellular extracts of MG1655 cells grown to different densities were loaded in lanes 5-9. The amount of cell extract loaded in each lane corresponded to 8.10×10^6 cells. Part B is a calibration curve to show the relationship between signal intensity and the mass of σ^{38} loaded. Part C shows a table in which the number of molecules of σ^{38} per cell is calculated for each growth point is calculated as in Figure S1.

A



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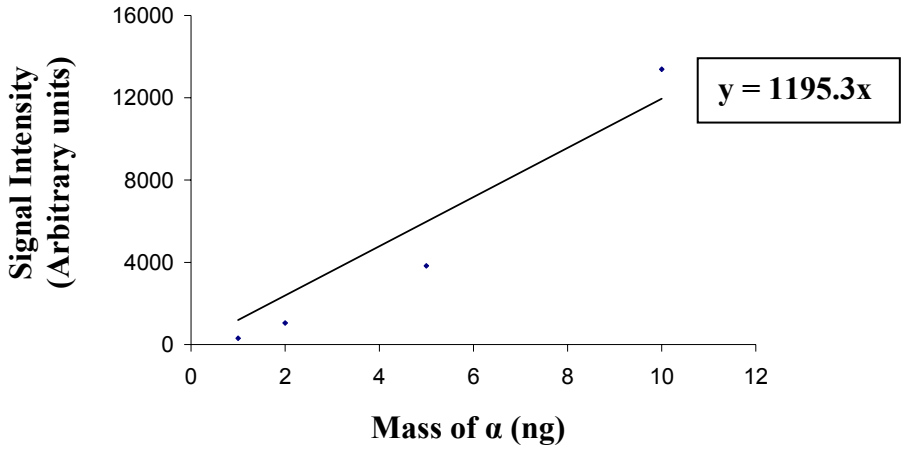


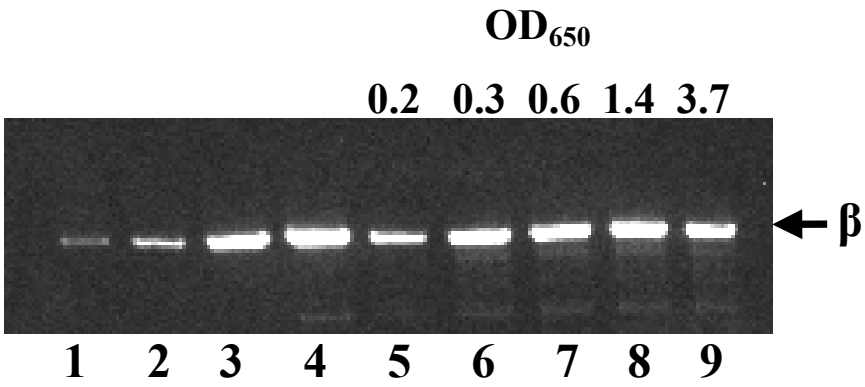
C

Sample	Mass of α (ng)	Number of molecules	Number of cells	Number of molecules per cell
MG1655 OD_{650} **0.3**	2.94	4.85×10^{10}	8.10×10^{6}	**5989**
MG1655 OD_{650} **0.5**	2.74	4.52×10^{10}	8.10×10^{6}	**5582**
MG1655 OD_{650} **0.7**	2.56	4.22×10^{10}	8.10×10^{6}	**5215**
MG1655 OD_{650} **1.6**	2.52	4.16×10^{10}	8.10×10^{6}	**5134**
MG1655 OD_{650} **3.8**	2.51	4.14×10^{10}	8.10×10^{6}	**5113**

Figure S4 Quantification of the RNA polymerase α subunit throughout growth of MG1655

Part A of the figure illustrates a western blot assay using mouse anti-α sera. Samples of purified holoenzyme were loaded onto an SDS-PAGE gel in lanes 1-4 (1 ng in lane 1, 2 ng in lane 2, 5 ng in lane 3 and 10 ng in lane 4), and cellular extracts of MG1655 cells grown to different densities were loaded in lanes 5-9. The amount of cell extract loaded in each lane corresponded to 8.10×10^{6} cells. Part B is a calibration curve to show the relationship between signal intensity and the mass of holoenzyme loaded. Part C shows a table in which the number of molecules of α per cell is calculated for each growth point is calculated as in Figure S1.

A



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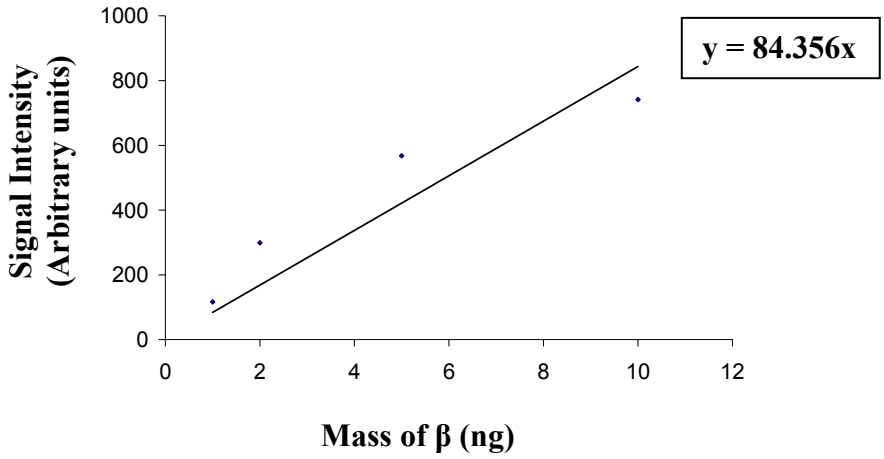

C

Sample	Mass of β (ng)	Number of molecules	Number of cells	Number of molecules per cell
MG1655 OD_{650} **0.2**	4.87	1.95×10^{10}	8.10×10^{6}	**2405**
MG1655 OD_{650} **0.3**	5.25	2.10×10^{10}	8.10×10^{6}	**2592**
MG1655 OD_{650} **0.6**	5.22	2.09×10^{10}	8.10×10^{6}	**2577**
MG1655 OD_{650} **1.4**	5.27	2.11×10^{10}	8.10×10^{6}	**2602**
MG1655 OD_{650} **3.7**	5.01	2.00×10^{10}	8.10×10^{6}	**2474**

Figure S5 Quantification of the RNA polymerase β subunit throughout growth of MG1655

Part A of the figure illustrates a western blot assay using mouse anti-β sera. Samples of purified holoenzyme were loaded onto an SDS-PAGE gel in lanes 1-4 (1 ng in lane 1, 2 ng in lane 2, 5 ng in lane 3 and 10 ng in lane 4), and cellular extracts of MG1655 cells grown to different densities, corresponding to 8.10×10^{6} cells, were loaded in lanes 5-9. Part B is a calibration curve to show the relationship between signal intensity and the mass of β. Part C is a table in which the number of molecules of β per cell is calculated for each growth point is calculated as in Figure S1.

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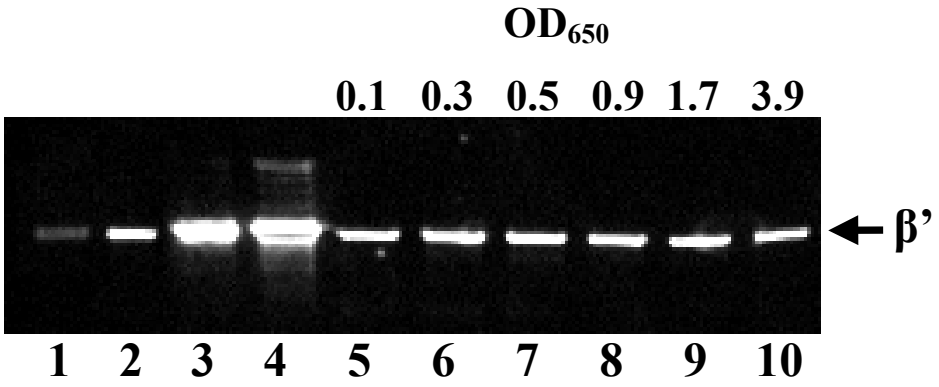

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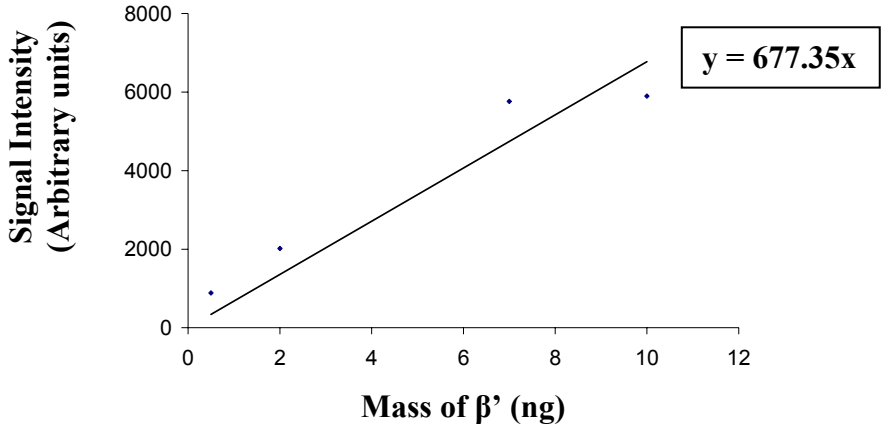


C

Sample	Mass of β' (ng)	Number of molecules	Number of cells	Number of molecules per cell
MG1655 OD_{650} **0.1**	5.28	2.05×10^{10}	8.10×10^{6}	**2530**
MG1655 OD_{650} **0.2**	5.53	2.15×10^{10}	8.10×10^{6}	**2649**
MG1655 OD_{650} **0.3**	5.32	2.06×10^{10}	8.10×10^{6}	**2549**
MG1655 OD_{650} **0.9**	5.39	2.09×10^{10}	8.10×10^{6}	**2582**
MG1655 OD_{650} **1.7**	5.29	2.05×10^{10}	8.10×10^{6}	**2534**
MG1655 OD_{650} **3.9**	5.23	2.03×10^{10}	8.10×10^{6}	**2506**

Figure S6 Quantification of the RNA polymerase β' subunit throughout growth of MG1655

Part A of the figure illustrates a western blot assay using mouse anti-β' sera. Samples of purified holoenzyme were loaded onto an SDS-PAGE gel in lanes 1-4 (500 pg in lane 1, 2 ng in lane 2, 7 ng in lane 3 and 10 ng in lane 4), and cellular extracts of MG1655 cells grown to different densities, corresponding to 8.10×10^{6} cells, were loaded in lanes 5-10. Part B is a calibration curve to show the relationship between signal intensity and the mass of β'. Part C is a table in which the number of molecules of β' per cell is calculated for each growth point growth point is calculated as in Figure S1.

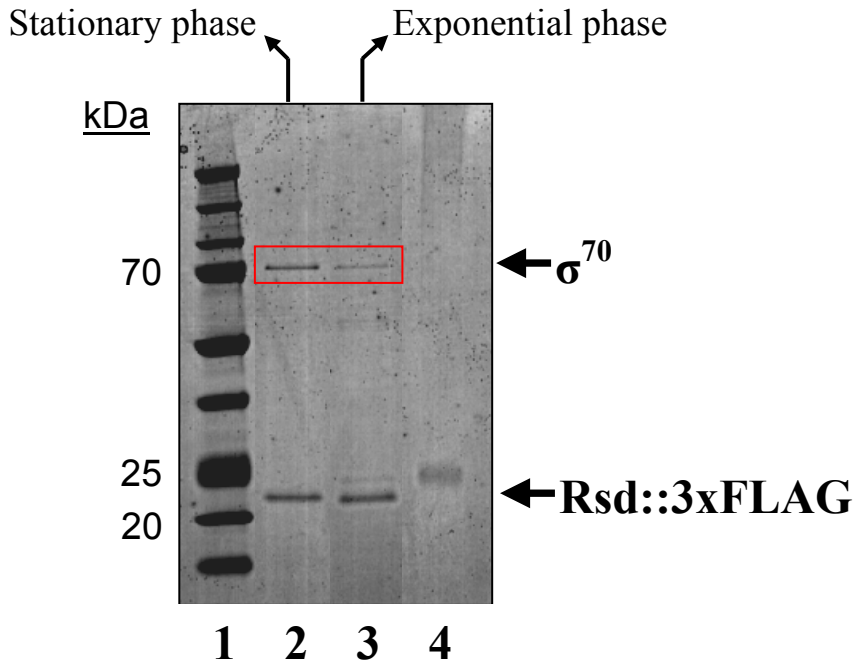


Figure S7 Immunoisolation assay to identify Rsd associated proteins

This figure shows an SDS-PAGE gel stained with Pierce GelCode Blue Stain Reagent. A broad range protein marker was loaded in lane 1 and the molecular mass values (kDa) of the standards used to calibrate the gel are shown. MG1655 rsd::3xFLAG cells were grown in LB and 1 g of cells was harvested from both exponential (OD_{650} 0.8) and stationary phase (OD_{650} 3.7). Cells were resuspended in an extraction buffer containing DNAse I and RNAse A and were then disrupted by sonication. Cellular lysates were added to 20 mg of magnetic beads (Dynabeads) coated with an anti-FLAG antibody to immunoisolate Rsd::3xFLAG and anything associated with it. These protein complexes were then eluted and analysed by SDS-PAGE. A 5 µl sample containing immunoisolates from stationary and exponential phase was loaded into lanes 2 and 3 respectively. The position of the bands corresponding to Rsd::3xFLAG and σ^{70} is indicated. Following the elution of Rsd::3xFLAG, a sample of the beads was loaded onto the gel (lane 4) to check that there was no protein still attached.

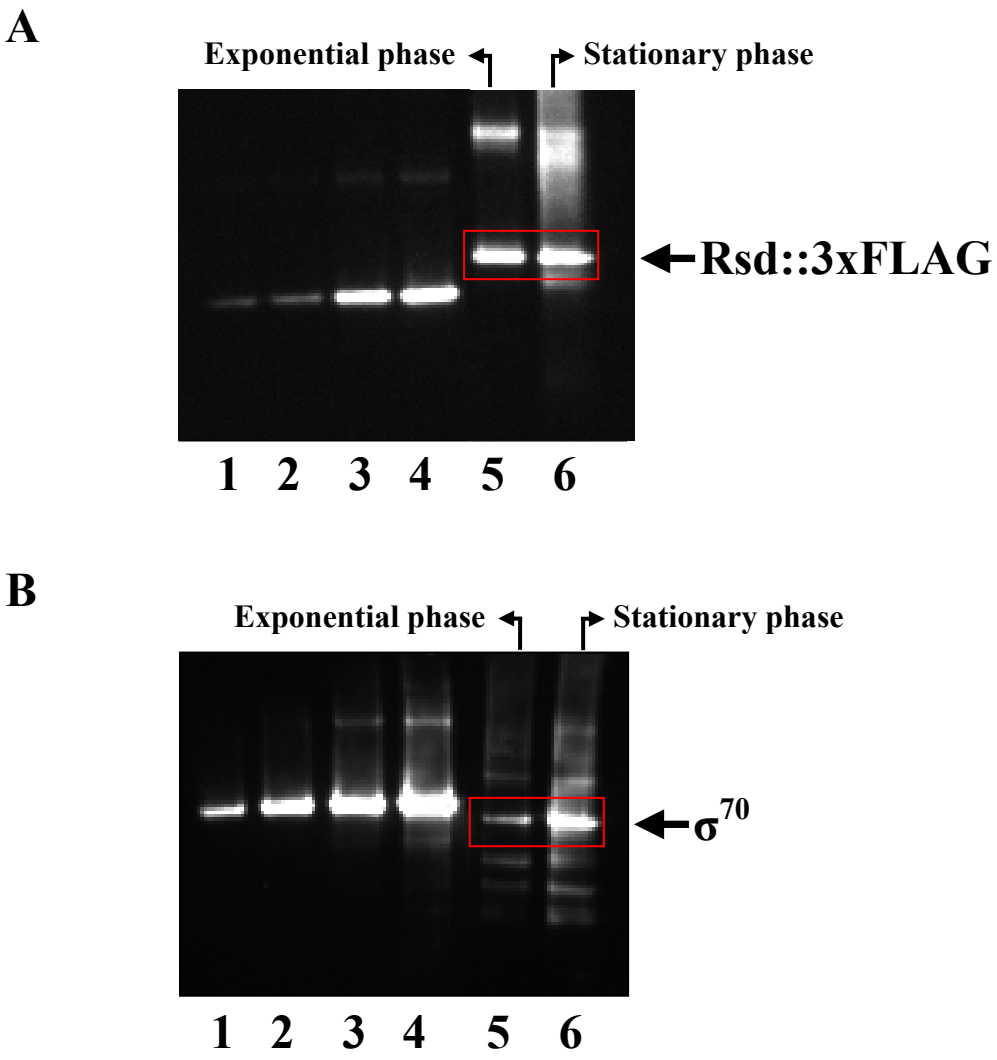
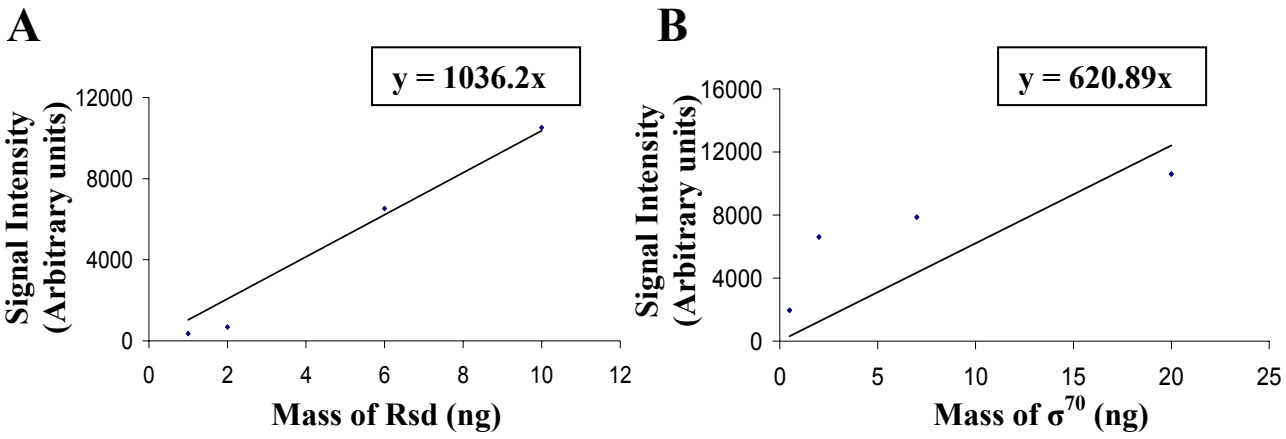


Figure S8 Rsd associations with σ^{70} during growth

This figure illustrates two western blot assays in which SDS-PAGE gels were probed with rabbit anti-Rsd sera (part A) or mouse anti-σ^{70} sera (part B). In part A, samples of purified Rsd of increasing concentration were loaded in lane 1 (1 ng), lane 2 (2 ng), lane 3 (6 ng) and lane 4 (10 ng). In part B, samples of purified σ^{70} of increasing concentration were loaded in lane 1 (500 pg), lane 2 (2 ng), lane 3 (7 ng) and lane 4 (20 ng). MG1655 *rsd::3xFLAG* cells were grown in LB and 1 g of cells was harvested from both exponential (OD_{650} 0.8) and stationary phase (OD_{650} 3.7). Cells were resuspended in an extraction buffer containing DNAse I and RNAse A and were then disrupted by sonication. The cellular lysates were then added to magnetic beads (Dynabeads) coated with an anti-FLAG antibody that immunoisolates Rsd::3xFLAG and any associated proteins. These protein complexes were eluted from the beads and a 5 µl sample containing the immunoisolates from either exponential or stationary phase was loaded into lanes 5 and 6 of both gels respectively. The position of the bands corresponding to Rsd::3xFLAG or σ^{70} is indicated.



C

Rsd::3xFlag	Exponential (OD_{650} 0.8)	Stationary (OD_{650} 3.7)
Mass of Rsd (ng)	5.87	7.68
Number of moles	3.26×10^{-13}	4.26×10^{-13}
Number of molecules	**1.96×10^{11}**	**2.57×10^{11}**

D

σ^{70}	Exponential (OD_{650} 0.8)	Stationary (OD_{650} 3.7)
Mass of σ^{70} (ng)	1.44	7.12
Number of moles	2.07×10^{-14}	1.02×01^{-13}
Number of molecules	**1.24×10^{10}**	**6.13×10^{10}**

Figure S9 Quantification of the amount of σ^{70} associated with Rsd during growth

The quantification shown in this figure is based on the two western blots shown in Figure S8. Parts A and B are calibration curves to show the relationship between the signal intensity and the mass of Rsd (part A) or σ^{70} (part B) loaded as standards onto the two SDS- PAGE gels. Parts C and D show tables in which the number of molecules of Rsd (part C) and σ^{70} (part D) eluted from the beads (following the immunoisolation) in exponential and stationary phase is calculated. In part C, the mass of Rsd (ng) presented in row 2 was estimated from the calibration curve in part A. The number of moles of Rsd given in row 3 was calculated by dividing the mass (g) by the M_r (18,000). The number of molecules was then determined by multiplying this number by Avogadro's constant (6.023×10^{23}). In part D, the mass of σ^{70} (ng) presented in row 2 was estimated from the calibration curve in part B. The number of moles of σ^{70} given in row 3 was calculated by dividing the mass (g) by the M_r (70,000). The number of molecules was then determined by multiplying this number by Avogadro's constant (6.023×10^{23}).

Protein	Number of molecules per cell	
	Exponential Phase (OD_{650} 0.3)	Stationary Phase (OD_{650} 3.0- 4.0)
Rsd	3318 ± 593	6242 ± 895
σ^{70}	7283 ± 913	7191 ± 898
σ^{38}	Not detectable	1614 ± 383
α	5428 ± 491	5377 ± 492
β	2493 ± 457	2535 ± 446
β'	2587 ± 458	2500 ± 485
RNA polymerase†	2598 ± 255	2574 ± 268

Table S1 Rsd, σ^{70}, σ^{38} and RNA polymerase subunit levels in MG1655 cells during exponential and stationary phase growth

The values shown are averages of measurements obtained from 3 independent quantitative western blot analyses done on three independent cultures. The western blot procedures and the subsequent calculations of the number of molecules of each protein are as described in Figures S1-S6. The calculated values for the number of molecules present at either OD_{650} 0.3 or OD_{650} 3.0-4.0 derive from averages of values from 3 independent experiments and the error is shown. To estimate the number of RNA polymerase molecules (†), an average was taken of all nine values from the 3 data sets for the core subunits (α, β, β').

Protein	Concentration (μM) of protein per cell	
	Exponential Phase (OD_{650} 0.3)	Stationary Phase (OD_{650} 3.0- 4.0)
Rsd	~5.5 μM	~10.4 μM
σ^{70}	~12.1 μM	~12.0 μM
σ^{38}	Not detectable	~2.7 μM
α	~9.0 μM	~9.0 μM
β	~4.2 μM	~4.2 μM
β'	~4.3 μM	~4.2 μM
Core RNA polymerase	~4.3 μM	~4.3 μM

Table S2 Concentrations of Rsd, σ^{70}, σ^{38} and RNA polymerase subunits and core enzyme during exponential and stationary phase growth of MG1655 cells

The average number of molecules per cell for each protein (Table S1) was converted into concentration by dividing the number of molecules by 1 x 10^{-15} (assumed to be the volume of an *E. coli* cell), and Avogadro's constant (6 x 10^{23}). This was converted into μM concentration by dividing by 1 x 10^{-6}.