Sigma 54 Levels and Physiological Control of the 

*Pseudomonas putida* Pu Promoter

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The cellular levels of the alternative sigma factor $\sigma^{54}$ of *Pseudomonas putida* have been examined in a variety of growth stages and culture conditions with a single-chain Fv antibody tailored for detection of scarce proteins. The levels of $\sigma^{54}$ were also monitored in *P. putida* strains with knockout mutations in *ptsO* or *ptsN*, known to be required for the C-source control of the $\sigma^{54}$-dependent Pu promoter of the TOL plasmid. Our results show that $\sim 80 \pm 26$ molecules of $\sigma^{54}$ exist per cell. Unlike that in relatives of *Pseudomonas* (e.g., *Caulobacter*), where fluctuations of $\sigma^{54}$ determine adaptation and differentiation when cells face starvation, $\sigma^{54}$ in *P. putida* remains unexpectedly constant at different growth stages, in nitrogen starvation and C-source repression conditions, and in the *ptsO* and *ptsN* mutant strains analyzed. The number of $\sigma^{54}$ molecules per cell in *P. putida* is barely above the predicted number of $\sigma^{54}$-dependent promoters. These figures impose a framework on the mechanism by which Pu (and other $\sigma^{54}$-dependent systems) may be amenable to physiological control.

Bacterial RNA polymerase (RNAP) holoenzymes are assembled by a common catalytic core enzyme that associates with a polypeptide (\(\sigma\)) conferring promoter recognition specificity. The majority of bacteria have alternative \(\sigma\) factors, most of which show homology with the major \(\sigma\) factor of *Escherichia coli* (\(\sigma^{70}\)) (34). A different class is composed of a unique member (\(\sigma^{54}\), encoded by *rpoN*) that differs both in amino acid sequence and mechanism of transcription activation (5). In essence, $\sigma^{54}$-RNAP holoenzyme forms a stable closed complex at the target promoter that is activated by a specialized family of regulators (20, 25) in a nucleotide (nucleoside triphosphate) hydrolysis-dependent manner (12, 26, 32).

A single copy of *rpoN* is found in the genome of many (but not all) bacterial species, including archetypal organisms such as *E. coli*, *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, and *Bacillus subtilis*. In *Pseudomonas putida*, *rpoN* exists as a single copy (24) and its expression is subject to negative autoregulation (18). A variety of biological functions are regulated by $\sigma^{54}$, although it appears that under favorable growth conditions these functions are dispensable since *rpoN* mutants are viable in all species tested except *Myxococcus xanthus* (17). The roles of $\sigma^{54}$ vary among various microbial species, a fact reflected in the expression profiles of the factor. While in *E. coli* the intracellular levels of $\sigma^{54}$ are constant throughout different growth stages (15), in *Caulobacter crescentus* the intracellular levels of $\sigma^{54}$ oscillate according to growth conditions and cellular differentiation (4). Although *P. putida* does not have a differentiation program, the number of niches in which this species thrives (water, soil, and plant roots) is so diverse (30) that bacteria must undergo major changes in their global physiological status during adaptation to the disparate habitats. In addition, many strains of *P. putida* have a versatile metabolism for utilization of recalcitrant carbon sources (including aromatic compounds such as xylene or phenol), and the genes for this metabolism are often under the control of $\sigma^{54}$-dependent promoters (e.g., the Pu promoter of TOL plasmid pWW0) (1). These promoters are subject to physiological regulation, becoming preferentially active at the stationary phase of growth (a phenomenon referred to as exponential silencing) (9), and modulation depending upon available carbon sources (i.e., C-source repression) (8, 10, 24, 27). Two genes, *ptsO* and *ptsN*, adjacent to *rpoN* in the *P. putida* chromosome, play a role in the C-source repression that glucose and glucan ameliorate the Pu promoter (11). Both *ptsN* and *ptsO* encode homologues of phosphoenolpyruvate:sugar phosphotransferase system family proteins IIA(Ntr) and NPR, respectively.

Several lines of evidence indicate that the physiological control of $\sigma^{54}$-dependent promoters is partially mediated through changes in $\sigma^{54}$ activity and/or protein levels. For instance, overexpression of $\sigma^{54}$ in *P. putida* allowed a partial relief of the exponential silencing of Pu (9). Also, modifications of the $\sim 12/-24$ motif of Pu that improve its similarity to the consensus $\sigma^{54}$ promoters have a positive effect on the transcription of this promoter in exponential phase (M. Carmona and V. Lorenzo, unpublished data). This suggests that recruitment of $\sigma^{54}$-RNAP may be a limiting step for Pu activation in vivo as it occurs in vitro (7). Further, activation of $\sigma^{54}$-dependent promoters in *E. coli* was found to depend upon the function of the specific protease FtsH, the lack of which can be compensated for by overproduction of the sigma (6).

The observations above highlight the importance of accurately quantifying the number of $\sigma^{54}$ molecules present in *P. putida* at the different stages of growth and in culture media that influence Pu promoter activity. Although such quantification was partially attempted in the past (9), the poor quality of the polyclonal antiserum employed flawed the conclusions and left unanswered the question of the number of $\sigma^{54}$ molecules per cell and the connection of $\sigma^{54}$ to Pu activity, in particular...
the modulation of $\sigma^{54}$ by C and N sources. By employing a dedicated phage antibody (Phab) displaying a single-chain Fv (scFv) antibody fragment with high affinity for $\sigma^{54}$ from \textit{P. putida}, we have determined accurately the number of $\sigma^{54}$ molecules in \textit{P. putida} cells at different growth stages and in various culture conditions. Our data indicate that $\sigma^{54}$ is one of the most invariable and least abundant cell proteins, thereby restricting the mechanisms that may account for the physiological control of $\sigma^{54}$.

**MATERIALS AND METHODS**

Strains, antibodies, and general procedures. Standard methods were used to purify, analyze, manipulate, and amplify DNA (2). The \textit{E. coli} strain XL-1 Blue (recA1 gyrA96 relA1 endA1 hsdM15 supE44 thi-1 lac [F' proAB lacY1 lacZAM15 Tn10 (Tc]) (Stratagene) was used as a host for bacteriophages and phagemids. Phagemid pPC2 bears the sequence of the high-affinity anti-$\sigma^{54}$ scFv named C2 assembled in vector pCANTAB-5Ehis (13) (details on scFv C2 are available upon request). scFv C2 specifically recognizes \textit{P. putida} $\sigma^{54}$ in enzyme-linked immunosorbent assays and in Western blots. Depending on the conditions employed for immunodetection, scFv C2 was produced as a distinct polypeptide or as a fusion with the $\sigma^{54}$ protein of the M13 phage. scFv-III hybrids were displayed as multiple copies on M13 particles (named Phab C2) by packaging the phage-fusions with the M13KO7pIII, Kmr (Progen) (28). scFv KT2442 and the \textit{P. putida} pttN::Km (10), pttO::Km (11), and rpoN::Km (19) mutants were grown at 30°C in the indicated media: Luria-Bertani broth (LB) (29), M9 plus CAA (M9 containing 0.2% [wt/vol] Casamino Acids; Difco) supplemented or not with 0.2% (wt/vol) glucose, high-nitrogen medium (M9 plus CAA supplemented with 0.2% sucinate), and low-nitrogen medium (modified M9 medium containing 2 mM NH$_4$Cl and supplemented with 0.2% sucinate). The last two media were supplemented with 0.05% (vol/vol) Triton X-100 to avoid cellular clumping.

Protein analyses. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with standard protocols by using the Mini-Protein system (Bio-Rad). Whole-cell protein extracts from \textit{Pseudomonas} cells were prepared by harvesting the cells (10,000 x g, 5 min) from cultures grown in the indicated media and resuspending the cell pellet in 100 μl of H$_2$O. Next, 100 μl of reducing 2X SDS sample buffer (120 mM Tris-HCl [pH 6.8], 2% [wt/vol] SDS, 10% [vol/vol] glycerol, 0.01% [wt/vol] bromophenol blue, 2% [vol/vol] 2-mercaptoethanol) was added to the samples, and the samples were heated at 100°C, sonicated briefly (~5 s), and centrifuged (14,000 x g, 10 min) to eliminate the DNA viscosity and any insoluble material (e.g., peptidoglycans). Loading was normalized by the number of cells determined as CFU per milliliter after plating in LB-agar (1.5% [wt/vol]) or by the total amount of protein (protein assay kit; Bio-Rad). Usually ~1.25 x 10$^8$ CFU or 10 μg of total protein was loaded per lane. Prestained standards (Kaleidoscope; Bio-Rad) were used as markers of known molecular weight for the SDS-PAGE. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) by using a semidry transfer apparatus (Bio-Rad). After protein transfer, the membranes were blocked for 2 h at room temperature (or for 16 h at 4°C) with MBT buffer (3% skimmed milk, 1% bovine serum albumin, and 0.1% Tween 20 in phosphate-buffered saline [PBS]).

Immunodetection techniques. For detection of $\sigma^{54}$ with the purified scFv C2, membranes with the blotted proteins were incubated with 10 ml of MBT buffer containing 500 ng of the antibody. Unbound scFvs were eliminated by four washing steps of 5 min in 40 ml of PBS and 0.1% (vol/vol) Tween 20. Next, anti-E-tag monoclonal antibody (MAB)-peroxidase (POD) conjugate (1:5,000 in MBT buffer; Amersham Pharmacia Biotech) was added to detect the bound scFvs. After 1 h of incubation, the membranes were washed four times with PBS and 0.1% (vol/vol) Tween 20 and the bound POD conjugates were developed with the BM chemiluminescence blotting substrate as described above. In order to standardize the protein amounts loaded in each case, duplicate blots were subjected to incubation with an anti-GroEL rabbit serum (1:5,000; kindly provided by J. M. Valpuesta, Centro Nacional de Biotecnología) and developed with anti-rabbit POD conjugate (1:5,000; Bio-Rad).

Quantification of $\sigma^{54}$. The intensity of light emitted by the protein bands in the membranes described above was quantified by employing the Quantity One software (Bio-Rad) and matched with a standard developed by using purified $\sigma^{54}$ protein (the kind gift of F. Bartels) run and processed under the same conditions. The absolute concentration of purified \textit{P. putida} $\sigma^{54}$ was determined by amino acid analysis for the standard curve. The protein sample was dried in a Speed-Vac (Beckman) and subsequently hydrolyzed in 6 N HCl-0.1% (vol/vol) phenol under vacuum conditions in a sealed glass tube for 24 h at 110°C. The amino acid analysis of the dried hydrolyzed protein sample was performed on a Beckman 6300 automatic analyzer to determine the amino acid composition as well as the protein concentration. Internal controls were performed with norleucine. This procedure allowed the accurate detection of 0.3 ng of $\sigma^{54}$.

**RESULTS AND DISCUSSION**

Rationale for quantification of $\sigma^{54}$ of \textit{P. putida} in vivo with a single-chain antibody. Antibody fragments assembled in M13 particles are particularly useful for the detection of proteins which are present in very small numbers in bacterial cells. This is because they can be either produced as distinct molecules or attached to phage particles (33). When the latter are employed as antibody-like agents, the use of a secondary anti-M13 coat antibody affords an extraordinary amplification from otherwise scarce target signals in the samples (22). Further, if the scFv is produced in an \textit{E. coli} strain subject to infection with a helper helper phage (28), then the resulting phage pool is composed of particles displaying multiple scFv units. This multiplicity the operative affinity and specificity of the antibody. On this basis, the antibody named scFv C2, targeted towards $\sigma^{54}$ of \textit{P. putida}, has been instrumental (both as Phab C2 and as purified scFv) in monitoring and accurately quantifying the levels of the factor under various physiological circumstances. This quantification was possible by simply matching the signals from cell extracts with a signal response standard set with purified $\sigma^{54}$ protein.

Levels of $\sigma^{54}$ in \textit{P. putida} cells at different growth stages. The levels of $\sigma^{54}$ in \textit{P. putida} cells were investigated at different points along the growth curve. To this end, \textit{P. putida} cells were grown at 30°C in rich liquid media (LB) and aliquots were taken at different time points (Fig. 1). The protein extracts obtained from the cells were analyzed by SDS–10% PAGE and Western blotting by using multivalent Phab C2 for detection (see Materials and Methods). Loading of the gels was normalized so that 10 μg of total protein was applied per lane. Detection of GroEL polypeptide, whose levels per cell remain constant under these conditions, was employed as an internal control for normalization in the Western blots (Fig. 1, bottom inset). As shown in Fig. 1, the levels of $\sigma^{54}$ in \textit{P. putida} cells were constant along the growth curve, at both exponential and stationary phases.

Nitrogen starvation does not affect $\sigma^{54}$ concentrations. In \textit{E. coli} cells, the activity of RpoN is essential for growth under nitrogen-limiting conditions. This is due to the requirement of the $\sigma^{54}$ for the transcriptional activation of $\text{glnA}$, which encodes the glutamine synthase, an enzyme responsible for the assimilation of ammonia at low concentrations (ca. 1 mM) (23). Similar to \textit{E. coli}, \textit{P. putida} rpoN mutant cells are unable to grow in media containing a low concentration of ammonia.
as the sole nitrogen source (19). Although low ammonia concentration does not induce rpoN transcription (18), we speculated that nitrogen starvation could otherwise affect the levels of the σ54 polypeptide in P. putida. Thus, protein extracts from P. putida cells grown in defined minimal media having low (2 mM NH₄Cl) or high (20 mM NH₄Cl) nitrogen content were analyzed by Western blotting (10 μg of protein was loaded per lane). These membranes were probed with the multivalent Phab C2 for detection of σ54 (top inset) or with a polyclonal antiserum against GroEL as an internal control (bottom inset). O.D. 600 nm, optical density at 600 nm.

These data demonstrate that nitrogen starvation does not affect the constant intracellular level of σ54 protein in P. putida.

Effect of ptsN and ptsO mutations on levels of σ54 in P. putida. Mutation of ptsN or ptsO of P. putida, two genes adjacent to and downstream of rpoN, influences in opposite ways the C-source control of the Pu promoter from pWW0. In a ptsN mutant, the activity of Pu is not repressed by glucose, whereas a ptsO mutant strain displays a phenotype of Pu repression even in the absence of glucose (10, 11). Experimental evidence suggests the existence of an equilibrium between the phosphorylated forms of PtsO, an NPr-like enzyme, and PtsN, a IIA(Ntr)-like enzyme. It is believed that glucose increases the share of phosphorylated forms of PtsN, which is in turn responsible for the repression of Pu by an undisclosed mechanism (10). In this context, we investigated whether P. putida ptsN and ptsO mutant strains grown in M9 plus CAA medium supplemented (top) or not (bottom) with 0.2% glucose.

Quantifying σ54 in P. putida cells. To accurately estimate the number of σ54 molecules per cell in P. putida, protein extracts derived from bacteria grown in different media (e.g., LB, M9 with high or low nitrogen concentration, and M9 plus CAA with and without glucose) were analyzed by quantitative Western blotting. In all cases, P. putida cells were harvested at stationary phase. In this assay, the signals obtained with purified scFv C2 against a series of twofold dilutions of purified σ54 (from 5 to 0.3 ng) were employed to generate a standard curve which allowed the precise determination of the amount of σ54 in the protein extracts normalized by numbers of CFU (Fig. 4). Quadruplicate experiments gave consistent results showing that ~80 ± 26 molecules of σ54 exist per cell in P. putida.

FIG. 1. Intracellular levels of σ54 in P. putida at different points along the growth curve. P. putida cells were grown in LB, and samples of these cultures were taken at different time points (filled squares). Whole-cell protein extracts derived from these cells were analyzed by Western blotting (10 μg of protein was loaded per lane). These membranes were probed with the multivalent Phab C2 for detection of σ54 (top inset) or with a polyclonal antiserum against GroEL as an internal control (bottom inset). O.D. 600 nm, optical density at 600 nm.

FIG. 2. Nitrogen starvation and σ54 levels in P. putida. Whole-cell protein extracts derived from P. putida cells grown in mineral media with high or low nitrogen content were obtained and analyzed by Western blotting as described in the legend to Fig. 1. Detection of σ54 and GroEL is shown in the top and bottom insets, respectively. O.D. 600 nm, optical density at 600 nm.

FIG. 3. Levels of σ54 in strains with mutations that affect the C-source regulation of Pu activity. Shown are Western blots to detect σ54 and GroEL (developed with Phab C2 and anti-GroEL serum, respectively) in whole-cell protein extracts (~10 μg was loaded per lane) obtained from samples, harvested at the indicated time points, of cultures of wild-type (wt) P. putida and the isogenic ptsN and ptsO mutant strains grown in M9 plus CAA medium supplemented (top) or not (bottom) with 0.2% glucose.

FIG. 4. Quantification of σ54 in P. putida cells. Protein extracts were prepared from P. putida KT2442 and the isogenic ptsN::Km and ptsO::Km strains, grown in M9 plus CAA medium supplemented or not with glucose (10 mM), and analyzed by immunoblotting with the multivalent Phab C2 as described above. The results from this experiment revealed that neither the presence of glucose nor the ptsO or ptsN mutation had an effect on the intracellular level of σ54 polypeptide in P. putida (Fig. 3). Therefore, these data prove that C-source repression of Pu in P. putida is unrelated to changes in the level of σ54.
without significant variation with the different media analyzed. Interestingly, these numbers are within the range of, but lower than, those reported for *E. coli* (~110 molecules/cell), which also remain roughly constant at exponential and stationary phases (15).

**σ^54** levels and physiological regulation of Pu. The results presented above demonstrate that the level of **σ^54** in *P. putida* is altogether constant at ~80 molecules/cell throughout any growth conditions. The number of **σ^54** molecules per *P. putida* cell is approximately twice the maximum number of **σ^54**-dependent promoters predicted in the genome of *P. putida* (~50 promoters; I. Cases et al., unpublished data). The low number of **σ^54** molecules is in contrast to the abundance of housekeeping sigma factor **σ^70** (~750 molecules/cell in *E. coli*) (14). Because of this, it is plausible that the available pool of the **σ^54**-containing form of the RNAP cannot saturate all **σ^54** promoters. Any condition that favors such an occupation may thus result in an increased output of the promoter under activation conditions. This framework, in which the artificial increase of **σ^54** levels relieves the physiological control of Pu (9), may simply reflect a higher occupation of the promoter by **σ^54**-RNAP. Under normal in vivo conditions, **σ^54**-RNAP alone fails to act on the Pu promoter and binding of the RNAP occurs only by virtue of the recruitment caused by the integration host factor (3, 7, 31). Sigma factor competition in stationary phase has been claimed as the major determinant of the physiological control of another related **σ^54**-RNAP promoter of *Pseudomonas* called Po (16, 21). Since *P. putida* has as many as 24 sigma factors, versus the 7 found in *E. coli* (24), the role of sigma competition in controlling the promoter output in vivo may be even more dramatic than anticipated (16, 21). A clear prediction of these notions is that promoters with high affinity for **σ^54**-RNAP may not undergo much physiological control whereas those with a lower affinity may be amenable to additional regulatory checks to promote **σ^54**-RNAP binding (Carmona and Lorenzo, unpublished). In any case, the low number of **σ^54** molecules may contribute decisively to the ability of the cells to rapidly adapt their metabolisms to changes in environmental conditions.

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