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Bacillus subtilis DesR Functions as a Phosphorylation-activated Switch to Control Membrane Lipid Fluidity*[S]

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Larisa E. Cybulski‡, Gloria del Solar§, Patricio O. Craig¶, Manuel Espinosa§, and Diego de Mendoza‡‖

From the ‡Instituto de Biología Molecular y Celular de Rosario (IBR), Consejo Nacional de Investigaciones Científicas y Técnicas, and Departamento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, 2002LRK-Rosario, Argentina, the §Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, 28040 Madrid, Spain, and the ¶Instituto Leloir, C1405BWE, Buenos Aires, Argentina

The Des pathway of *Bacillus subtilis* regulates the synthesis of the cold-shock induced membrane-bound enzyme Δ5-fatty acid desaturase (Δ5-Des). A central component of the Des pathway is the response regulator, DesR, which is activated by a membrane-associated kinase, DesK, in response to a decrease in membrane lipid fluidity. Despite genetic and biochemical studies, specific details of the interaction between DesR and the DNA remain unknown. In this study we show that only the phosphorylated form of protein DesR is able to bind to a regulatory region immediately upstream of the promoter of the Δ5-Des gene (P*des*). Phosphorylation of the regulatory domain of dimeric DesR promotes, in a cooperative fashion, the hierarchical occupation of two adjacent, non-identical, DesR-P DNA binding sites, so that there is a shift in the equilibrium toward the tetrameric active form of the response regulator. Subsequently, this phosphorylation signal propagation leads to the activation of the *des* gene through recruitment of RNA polymerase to P*des*. This is the first dissected example of a transcription factor functioning as a phosphorylation-activated switch for a cold-shock gene, allowing the cell to optimize the fluidity of membrane phospholipids.

All organisms must communicate with their environment to survive. Bacterial cells monitor external conditions and process this information to give the most appropriate response. Two-component regulatory systems have emerged as a paradigm for adaptive responses. In its simplest form, a two-component system contains a sensor (histidine kinase) and a response regulator (often a transcriptional activator) (1). Changes in the environment result in phosphorylation of the sensor followed by transphosphorylation onto the response regulator (1, 2). Although kinase-response regulator pairs of this type were frequently reported as governors of a wide variety of pathways in response to a myriad of signals (3–5), the requirement of this system to control gene expression during cold-shock has only recently been discovered in *Bacillus subtilis* (6) and cyanobacteria (7).

Cold shock is a stress condition that adversely affects the growth of poikilothermic organisms, such as bacteria and plants. Thus, understanding the mechanisms by which these organisms perceive low temperature signals and transmit this information to the cellular machinery to activate adaptive responses is of fundamental importance to biology.

Bacteria and most (if not all) poikilothermic organisms have to remodel the membrane lipid composition to survive at low temperatures. *B. subtilis* (6) and *Synechocystis* (7) respond to a decrease in the ambient growth temperature by introducing double bonds into the acyl chains of their membrane phospholipids by membrane-bound acyl lipid desaturases. This post-synthetic modification of the saturated acyl chains seems to be designed to ameliorate the effect of temperature changes on the physical state of membrane phospholipids. In *Synechocystis* it was found that inactivation of two histidine kinases moderates the low-temperature induction of the genes coding for Δ6 and ω-3 desaturase (7). However, the transcriptional regulators of these genes were not yet identified. A better understood example of perception and transduction of low temperature signals is the Des pathway of *B. subtilis*, which regulates the expression of the acyl lipid desaturase, Δ5-Des. This pathway responds to a decrease in growth temperature by enhancing the expression of the *des* gene coding for Δ5-Des (8–11). The Des pathway is uniquely and stringently controlled by the two-component system, DesK/DesR (6). DesK is a histidine kinase located in the membrane and DesR is a 22.18-kDa cytoplasmic response regulator that binds specifically to the promoter region it controls. Response regulators have been classified according to similarities in the DNA binding domain. DesR belongs to the NarL group, in which the C-terminal binding domain contains a helix-turn-helix motif (2). Based on the two-component system paradigm and on previous work, we have proposed that the two proteins communicate via a series of phosphorylation and phosphotransfer reactions. DesK senses a decrease in membrane lipid fluidity and is autophosphorylated by intracellular ATP at His-188 (12, 13). We have recently demonstrated that DesK-P is able to phosphorylate DesR (13) so we suggested that this covalent modification of DesR allows its interaction with the *des* regulatory region, leading to activation of *des* transcription. This assumption was based on the fact that no bacterial response regulator has yet been found to be active in the unphosphorylated form, although some response regulators, such as yeast SSK1 I, are inactive in the phosphorylated form (14). Although genetic studies have

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[S] The on-line version of this article (available at http://www.jbc.org) contains Figs. S1–S5 and Tables I and II.

‖ Career investigator from Consejo Nacional de Investigaciones Científicas y Técnicas and an International Research Scholar from the Howard Hughes Medical Institute. To whom correspondence should be addressed: Instituto de Biología Molecular y Celular de Rosario (IBR) and Departamento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2002LRK-Rosario, Argentina. Tel.: 54-341-4351235; Fax: 54-341-4390465; E-mail: diegonet@citynet.net.ar.

shown that DesR is absolutely necessary for *des* expression, it is unknown whether DesR is able to stimulate transcription *per se* or it is required to stimulate the synthesis or activity of another factor necessary for *des* transcription.

In the present study we have examined how phosphorylation influences the ability of DesR to bind to the *des* promoter (P*des*).[1] Our results show that only the phosphorylated form of DesR, DesR-P, is able to bind to the regulatory region it controls. In addition, we demonstrate that DesR is a dimer in solution that tends to tetramerize upon phosphorylation, which could account for cooperative interaction between molecules of DesR-P bound at adjacent DesR regulatory binding sites at P*des*. We show that DesR-P activates *des* transcription through recruitment of the RNA polymerase (RNAP) to the *des* promoter. A model for DesR-P promoter recognition has emerged from our results that we discuss in the context of transcriptional regulation of membrane fluidity homeostasis.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmid, and Strain Constructions—The bacterial strains used in this work are listed in Supplementary Materials Table I. β-Galactosidase was assayed as previously described (15). To mutate the IR-L inverted repeat of the regions that were protected in footprinting experiments (Fig. 4), oligonucleotides I, II, III, and IV were used for the amplification of two overlap-extension PCR (see Supplementary Materials Table II). A mixture of these PCR products was used as DNA template for another PCR using oligonucleotides I and VII (16). The 301-bp amplification product was cloned into the integrational vector pJM116 (17) to generate pLC118. To mutate IR-R, oligonucleotides V and VI were utilized instead of II and III, and the resulting plasmid was pLC119. These plasmids were introduced into the *amyE* locus of strain JH642 giving strains LC118 and LC119, respectively.

The same strategy was used to mutate the putative phosphorylation site Asp-54 with Ala, using oligonucleotides VIII, IX, X, and XI. The amplification product was cloned into plasmid pGS791[2] under the xylose promoter, yielding plasmid pLC90. This plasmid was integrated ectopically in the *thrC* locus of a *B. subtilis* strain that lacks DesR and contains a fusion of the *lacZ* reporter gene to the *des* promoter (AKP9, see Ref. 6) yielding strain LC90. Wild type *desR* was amplified using oligonucleotides VIII and XI, and was cloned in the same way as *desRD54A*, giving strain LC89. All plasmids were sequenced to confirm the introduction of mutations.

Gel Mobility Shift Assays—DNA fragments including the wild type or different *des* promoter variants were prepared by PCR using primers $XIII_{(PECO895)}$ and $VII_{(BAMO)}$ (Supplementary Materials Table II). The assay was performed as described previously (6). When phosphorylated DesR was used in the reaction, 6 μM DesR was incubated for 20 min at 0 °C in a mixture containing 50 mM Tris-HCl, pH 8, 5 mM $MgCl_2$, 0.5 mM EDTA, 1.25 mM dithiothreitol, 10% glycerol, and 50 mM acetyl phosphate.

For the ternary complex formation, the DNA fragment was prepared using the primers XIV and XV (Supplementary Materials Table II). The RNAP Holoenzyme (Epicenter) was added at 100 nM and incubated 30 min at 25 °C before running the gel. When heparin was included in the reaction, it was added for 5 min, at 60 μg/ml, after the incubation with the RNAP.

$OH^{\cdot}$ Footprinting Assays—Hydroxyl radical treatment was performed essentially as described (18). A typical reaction mixture contained 2.6 nM 5′-end-labeled DNA fragment extending from −167 to +10 relative to the *des* transcription start site, DesR-P (300 or 1000 nM), 25 mM Tris-HCl, pH 8, 1 mM dithiothreitol, 0.25 mM EDTA, 4 mM $MgCl_2$, and 200 ng of poly(dI-dC). After 30 min incubation at room temperature, 1 mM sodium ascorbate, H_2O_2 to 0.03%, and $Fe(EDTA)^{2-}$ (final concentrations of 18 and 36 μM for Fe(II) and Na_2EDTA, respectively) were added, and the incubation was continued for 1 min at the same temperature. The reactions were stopped by the addition of a thiourea/EDTA solution (final concentrations of 9.5 mM thiourea and 1.7 mM EDTA). Mixes were run in a non-denaturing 5% polyacrylamide gel. The gel was exposed to an autoradiographic film for 5 min, and complexes I and II were cut out of the gel. The DNA was eluted, precipitated, loaded on denaturing 8% polyacrylamide sequencing gels, and run together with the products of sequencing reactions of Maxam and Gilbert (G + A) obtained from the same labeled DNA. The band pattern was visualized using a Fuji Film Phosphorimager.

Separation of DesR from DesR-P—DesR or DesR phosphorylated *in vitro* with acetyl phosphate (Ac-P) for 20 min were injected on a C4 reversed phase Vydac column, using an ÄKTA Liquid chromatograph. Eluent A was 0.1% trifluoroacetic acid, 20% acetonitrile in water; eluent B was 0.1% trifluoroacetic acid, 60% acetonitrile in water. The sample was eluted with a gradient that varied from 80 to 0% A, over 40 min. Eluent B started at 20% and went to 100% over the same interval. The flow rate was 1 ml/min.

Analytical Ultracentrifugation—Analytical ultracentrifugation studies were performed in a Beckman XL-A (Beckman Instruments Inc.) equipped with a UV-visible optic system inside the microcentrifuge. Sedimentation equilibrium experiments were carried out at 12,000 rpm and 20 °C in Epon eight-channel centerpieces with protein loading concentrations in the range of 1–12 μM. DesR was equilibrated in a buffer containing 50 mM Tris-HCl, pH 8, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM dithiothreitol (and 50 mM Ac-P when indicated). Radial absorption scans at 231, 275, and 280 nm were taken after the sedimentation equilibrium was reached. Baseline offsets were determined by high-speed centrifugation (42,000 rpm).

Experimental data were analyzed using the program Sednterp version 1.03 XLAEQ and EQASSOC (Beckman; 19–20), using a partial specific volume for DesR of 0.7449 ml g^{-1}. This value was determined on the basis of its amino acid residue composition. The values of extinction coefficients at 280 nm were calculated from the known tryptophan and tyrosine residue contents.

Determination of the Molecular Weight of DesR by Static Light Scattering—The average molecular weight (M_r) of wild type DesR and its mutant DesRD54A treated and non-treated with Ac-P were determined on a Precision Detector PD2010 light scattering instrument tandemly connected to an high performance liquid chromatography system and a LKB 2142 differential refractometer. The samples were loaded on a Sephadex G-25 (1 ml) column and eluted with 50 mM Tris-HCl, pH 8, 0.3 M NaCl, 0.5 mM dithiothreitol, at a flow rate of 0.3 ml/min. The 90° light scattering and refractive index signals of the eluting material were recorded on a PC computer and analyzed with the Discovery32 software supplied by Precision Detectors. The 90° light scattering detector was calibrated using bovine serum albumin (66.5 kDa) as a standard.

In Vitro Transcription Assays—*In vitro* transcription reactions (50 μl) were performed in a buffer containing 40 mM Tris-HCl, pH 8, 10 nM $MgCl_2$, 150 mM KCl, 0.1 mM EDTA, and 5% glycerol. A DNA fragment extending from position −177 to position +43 was prepared by PCR using oligonucleotides PA and PC (Supplementary Table II). The DNA (5 nM) was incubated first with DesR-P (300 nM) for 20 min at 25 °C, and then 10 min with RNAP (0.2 units) at the same temperature. The mixture was then treated with 10 μg/ml heparin for 3 min at 37 °C. Reactions were started by the addition of 5 μl of a solution 2 M of the four NTPs containing 0.5 μM [α-^{32}P]UTP (400 Ci/mmol; 10 μCi/μl). After 5 min of incubation at 37 °C, the reaction was terminated by the addition of sodium acetate, pH 7, to 300 mM, EDTA to 15 mM, and tRNA to 100 μg/μl, and the nucleic acids were precipitated by the addition of 250 μl of 100% ethanol. The products were fractionated by electrophoresis on 8% polyacrylamide gels containing 8 M urea.

Equation to Calculate the Relative Dissociation Constant of Regulator-DNA Complexes I and II—K_{dI} and K_{dII} represent the dissociation constants for specific complexes I and II, respectively. f_{maxCI} represents the fractional maximum level of complex I attained in the reactions (21).

$$\mathrm{DNA} + \mathrm{DesR} \underset{K_{dI}}{\rightleftharpoons} \mathrm{CI} \xrightarrow[K_{dII}]{} \mathrm{CII} \qquad \text{(Eq. 1)}$$

(DesR)

$$\frac{K_{dI}}{K_{dII}} = \left[\frac{1}{f_{maxCI}} - 1\right]^2 \qquad \text{(Eq. 2)}$$

RESULTS

Phosphorylation of DesR Increases the Affinity of Binding to Its Target DNA—We wished to examine how phosphorylation influences the ability of DesR to bind to a DNA region spanning P*des* by electrophoretic mobility shift assays (EMSA) using DesR or DesR-P, this latter phosphorylated *in vitro* with Ac-P.

[1] The abbreviations used are: P*des*, *des* promoter; RNAP, RNA polymerase; EMSA, electrophoretic mobility shift assay; Ac-P, acetyl phosphate; CI and CII, complexes I and II; RA and RB, regions A and B; DR, direct repeat.

[2] G. Schujman, personal communication.

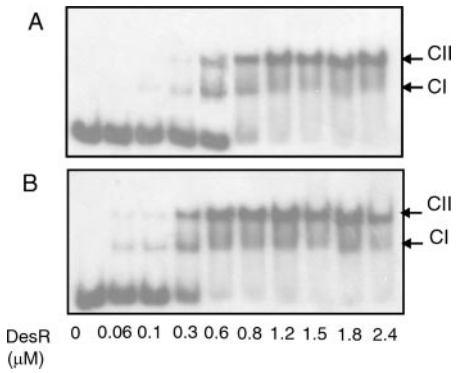

FIG. 1. **Gel mobility shift assay showing the binding of DesR or DesR-P to P*des*.** The radiolabeled P*des* probe was incubated with increasing amounts of DesR (*A*) or DesR phosphorylated *in vitro* with acetyl phosphate (*B*). *CI* and *CII* indicate the fast and slow migrating DesR-DNA complexes, respectively.

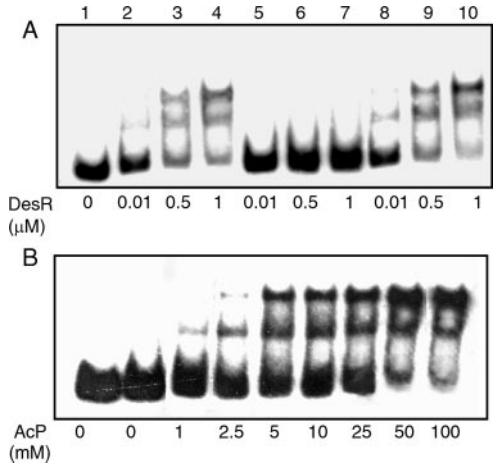

FIG. 2. *A*, gel mobility shift assay of the radiolabeled P*des* probe incubated with freshly purified DesR (*lanes 2–4*), DesR dephosphorylated by incubation at 25 °C for 12 h (*lanes 5–7*), or dephosphorylated DesR incubated with 50 mM Ac-P (*lanes 8–10*). *B*, gel mobility shift assay of the radiolabeled P*des* probe incubated with 800 nM dephosphorylated DesR treated with increasing amounts of Ac-P.

To this end, a PCR-amplified [α-^{32}P]dATP-labeled 216-bp DNA fragment extending from positions −187 to +29, relative to the *des* transcription start site, was incubated with purified DesR or DesR-P proteins. The results showed that P*des* exhibited changes in its electrophoretic mobility in the presence of either DesR (Fig. 1*A*) or DesR-P (Fig. 1*B*); however, when DesR-P was used, lower protein concentrations were sufficient to obtain the same fraction of complexed DNA. Phosphorylated DesR showed binding to the *des* promoter at concentrations as low as 0.06 μM. At this concentration a fast migrating complex I (CI) was mainly observed. Increasing DesR-P concentration enhanced progressively the formation of the slow-migrating complex II (CII; Fig. 1*B*). At 0.6 μM, CII is clearly the predominant protein-DNA complex. Formation of two different complexes, CI and CII, suggested to us the existence of two binding sites for the response regulator. DesR without Ac-P treatment was also able to bind to P*des*, but higher concentrations were needed to form CI and CII (Fig. 1*A*).

DesR Phosphorylation Site Is Asp-54—Sequence comparative analysis of bacterial response regulators using multiple sequence alignment with hierarchical clustering (Multalin) indicated that Asp-54 of DesR should be the residue that receives the phosphoryl group from the cognate kinase, DesK. To determine whether this putative phosphorylation site, Asp-54, was involved in DesR activity, it was substituted with Ala using site-directed mutagenesis. DNA fragments carrying either *desR*$^+$ (wild type) or *desR* encoding the mutant protein (*desRD54A*) were provided in *trans* into a *desR* null mutant carrying a fusion of the *lacZ* reporter gene to the *des* promoter. The expression of wild type *desR*$^+$ allowed stimulation of *des* transcription upon a temperature downshift, whereas the expression of *desRD54A* could not stimulate transcription of the *des-lacZ* fusion under the same conditions (Supplemental Materials Fig. S1), indicating that residue Asp-54 is crucial for *in vivo* DesR transcriptional activity. Confirming this conclusion, we have determined that autophosphorylated DesK serves as a phosphodonor of the effector protein DesR, but cannot transfer the phosphoryl group to DesRD54A (data not shown).

The Unphosphorylated Form of DesR Is Unable to Bind to the des Promoter—Some response regulators such as *B. subtilis* PhoP bind to its target promoters either in the unphosphorylated or in the phosphorylated form. However, the unphosphorylated form of PhoP is unable to activate transcription (22). As shown in Fig. 1*A*, DesR binds to P*des* even without previous treatment with Ac-P, although *in vivo* experiments (described in Supplemental Materials Fig. S1) indicated that phosphorylation of Asp-54 is crucial for DesR activity. This suggested that DesR behaved similarly to PhoP. If this were the case, DesRD54A should bind to P*des* in EMSA, in the same way as unphosphorylated DesR, but treatment of DesRD54A with Ac-P should not increase its binding affinity. To evaluate this possibility, binding of purified DesRD54A to P*des* was tested using EMSA. Surprisingly, DesRD54A was unable to bind to P*des* at any tested concentration even when treated with Ac-P (Supplemental Materials Fig. S2*A*). This result could be attributed to: (i) the unphosphorylated form of DesR does not bind to P*des*, and the binding activity observed in the experiment shown in Fig. 1*A* was because of nonspecific phosphorylation of DesR during its overproduction in *Escherichia coli,* or (ii) the point mutation Asp-Ala in DesRD54A provoked a conformational change that abolished the binding of the mutated response regulator to P*des*. To distinguish between these two possibilities, DesR purified from *E. coli* was subjected to reverse phase high performance liquid chromatography (Supplemental Materials Fig. S2*B*). DesR showed two peaks that correspond to the phosphorylated and unphosphorylated form of the regulator. When DesR extracted from *E. coli* was incubated with Ac-P, the peak corresponding to DesR-P increased considerably (Supplemental Materials Fig. S2*C*). This result indicates that about 50% of His-tagged DesR purified from *E. coli* is phosphorylated, and that this form could be responsible for the binding activity of DesR without Ac-P treatment in the EMSA assays showed in Fig. 1*A*. It is worth mentioning that circular dichroism analysis showed that DesRD54A presents the same global secondary structure as wild type DesR (Supplemental Materials Fig. S2*D*), supporting the conclusion that this mutant regulator does not bind to P*des* because it is unable to be phosphorylated in the conserved aspartate by Ac-P, rather than by a structural change produced by the mutation. To demonstrate directly that the unphosphorylated form of DesR does not bind to its target promoter, we took advantage of the observation that DesR-P is completely dephosphorylated after incubation at 25 °C for 12 h (13). We tested by EMSA the retardation of P*des* in the presence of untreated DesR (as it elutes from the nickel-nitrilotriacetic acid column), DesR dephosphorylated by incubation at 25 °C for 12 h, or DesR dephosphorylated at 25 °C and phosphorylated again by treatment with Ac-P. Fig. 2*A* shows that untreated DesR forms two complexes (*lanes 2–4*), whereas DesR dephosphorylated at 25 °C is unable to bind to P*des* (*lanes 5–7*). As expected, when this unphosphorylated DesR was incubated with Ac-P, it recovered the DNA binding activity (*lanes 8–10*). From now on, we

will call "unphosphorylated DesR" the protein that was incubated 12 h at 25 °C. In addition, incubation of unphosphorylated DesR with increasing concentrations of Ac-P resulted in a similar enhancement of the binding of the response regulator, reaching a maximum at 50 mM of the phosphoryl donor (Fig. 2*B*). From the above results we can draw two conclusions: (i) the extent of phosphorylation of DesR determines its binding capacity, and (ii) unphosphorylated DesR is unable to bind to its target promoter.

It should be mentioned that when DesRD54A was incubated with 50 mM Ac-P, and then subjected to high performance liquid chromatography, we detected that a secondary site(s) of the protein was phosphorylated (data not shown). Nevertheless, as shown in Supplemental Materials Fig. S2*A*, phosphorylation of this secondary site was unable to stimulate DesRD54A binding to P*des*.

DesR Is a Dimer in Solution and Tetramerizes upon Phosphorylation—Phosphorylation promotes the formation of dimers or even oligomers of many response regulators; however, other response regulators do not change their association state upon phosphorylation (23–26). The existence of two DesR-P·DNA complexes (Figs. 1 and 2) indicates that phosphorylation of DesR favors the binding of more than one subunit of the protein to the *des* promoter. To study the association state of DesR or DesR-P, we performed sedimentation equilibrium experiments by analytical ultracentrifugation. In the case of DesR-P it was necessary to define the required experimental conditions to stabilize the phosphorylated form during the course of the sedimentation assay. Because the half-life of DesR-P at 25 °C is about 90 min (13), a high steady state of phosphorylation was maintained *in situ* by the inclusion of an excess of Ac-P in the sample subjected to analytical ultracentrifugation. Ultracentrifugation runs were performed either with unphosphorylated DesR or with DesR-P at concentrations in the range of 1 to 12 μM. Unphosphorylated DesR behaved as a dimer in solution at every tested concentration, because all the experimental data fit the Sednterp predicted curve for a dimer (Supplemental Materials Fig. S3*A*). When DesR was subjected to analytical ultracentrifugation in the presence of Ac-P, its hydrodynamic behavior changed considerably, especially at the highest protein concentration, at which the tetramer became the most abundant species (Supplemental Materials Fig. S3*B*), although the best fit includes constants for higher order multimers. This suggests that phosphorylation of DesR promotes new interaction between DesR dimers. Plots of the buoyant molecular mass *versus* protein concentration (1–12 μM) indicated that at low protein concentrations, the molecular mass of DesR-P corresponded to a dimer (Supplemental Materials Fig. S3*C*). As the levels of DesR-P increased, the mass of the species became larger, reaching the mass of a tetramer. This is a characteristic behavior of a system in equilibrium; otherwise dilution would not affect the average molecular mass of the species.

To confirm the hydrodynamic properties of DesR obtained by analytical ultracentrifugation and to investigate the tetramerization phenomenon in the absence of continuous phosphorylation by Ac-P, we resort to static light scattering experiments. To this end, solutions of unphosphorylated DesR or DesR incubated with Ac-P were dialyzed for 20 min to remove the phosphorylating agent. Then, the total scattered intensity at 90° was examined. The average molecular weight of DesR was 41,553, and that of DesR-P was 71,834, corresponding to 1.94 and 3.36 units of DesR, respectively. The latter fractional number suggested that in this condition phosphorylation with Ac-P does not reach completion, and that about 70% of DesR was phosphorylated on this sample. Control experiments with DesRD54A showed an average molecular weight corresponding to a dimer regardless of the Ac-P treatment. Taken together, these experiments demonstrated that DesR is a dimer in solution that mainly forms tetramers upon phosphorylation.

High-resolution Contacts of DesR on Its Target—Previous DNase I footprinting assays have shown that DesR binds to a DNA sequence that extends from positions −28 to −77 relative to the *des* transcription start site, generating 5 protected regions on each strand (6). Two inverted repeats (5′-TCAT-3′) separated by 9 nucleotides were found in the center of the protected region. To obtain a high resolution profile of the contacts between DesR-P and its target DNA we resorted to hydroxyl radical footprinting analysis. To favor the formation of CI (faster migrating complex) or CII (slower migrating complex), 300 or 1000 nM DesR-P were used in the reaction, respectively. After the Fenton reaction (see "Materials and Methods"), the mixtures were run in a typical non-denaturing EMSA gel. Protein-DNA complexes were excised out of the gel, the DNA was purified and subjected to electrophoresis on denaturing sequencing gels. Fig. 3*A* shows typical results of the $OH^{\cdot}$ cleavage in the absence and presence of DesR-P for both coding and non-coding strands. Four out of the five protected regions observed in the DNase I footprinting assays were also detected by hydroxyl radical. Footprints 1 and 2, as well as 3 and 4 on each strand were spaced 9–11 nucleotides, footprints 2 and 3 were spaced 14 and 12 nucleotides on the coding and non-coding strand, respectively. The distribution of bases whose deoxyriboses are protected from $OH^{\cdot}$ attack by bound DesR-P at the *des* promoter is depicted in Fig. 3*B*. Considering a helical periodicity of 10.5 bp/turn, it is clear that DesR-P contacts with the DNA backbone at the same face of the DNA helix in each one of two different regions (RA and RB), although the contacts in RB are shifted 70° clockwise from the DesR-P contacts in RA (Fig. 3*B*). PhosphorImager quantification of the $OH^{\cdot}$ footprinting assays (Fig. 3*A*) revealed that DesR-P associated to CI protects more efficiently DNA region A of P*des*, whereas the response regulator associated to CII protects regions A and B (Supplemental Materials Fig. S4). In CI, DesR-P seems to protect RB slightly, although this could be due either to contamination between complexes or to a small population of CI that has DesR-P bound to RB. In addition to the inverted repeat (5′-TCAT-3′) previously identified (6) in the center of the entire region contacted by the response regulator and *boxed* in Fig. 4*A*, inspection of region A revealed a 6-bp motif, 5′-ATGACA-3′ (*IR-L*, *left*), separated 2 bp apart from its counterpart 5′-TGTCAT-3′ (*IR-R*, *right*; Fig. 4*A*). As suggested by the PhosphorImager analysis of the DesR-P contacts in CI and CII (Fig. 3), RA could be the primary binding site for a DesR-P dimer because this region was occupied by DesR at low protein concentrations. As shown in Fig. 4*A*, low sequence homology between DesR-P binding regions A and B was found. The palindromic motif is absent in RB, although the four first nucleotides of IR-L (5′-ATGA-3′) are present in this region. We called this incomplete motif, direct repeat (DR).

To determine whether the putative recognition motifs identified by $OH^{\cdot}$ footprinting are indeed necessary for DesR-P/DNA interactions, promoter variants carrying a deletion affecting RA and RB (D22) or mutations in the IR-L, IR-R, and DR (Fig. 4*B*) were analyzed by EMSA. P*des*IR-L and P*des*IR-R, in which the IRs L or R (5′-ATGACA-3′) were mutated to 5′-TCTAGA-3′, were unable to form any complex with DesR-P, except at very high concentrations when a poor high molecular weight complex (CII) was formed (Fig. 4*C*). These results show that mutations in one of the IRs of region A prevent the interaction of the response regulator with regions A and B. P*desDR*, in which the sequence 5′-CATGAT-3′ including the DR of RB

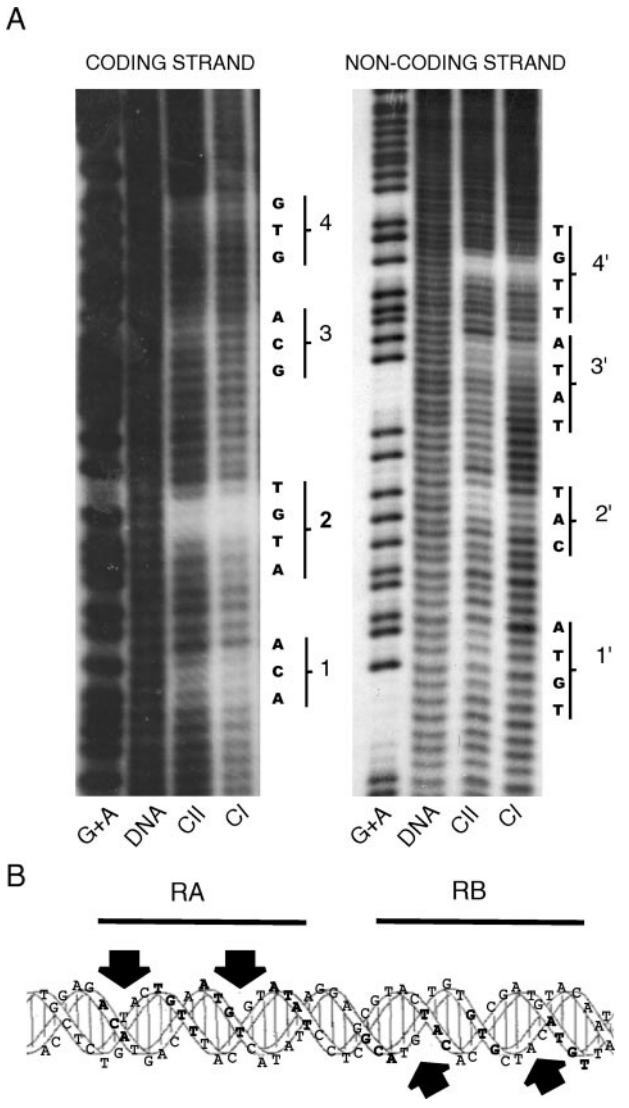

FIG. 3. *A*, HO˙ footprinting analysis of the DesR-P·P*des* complex. The P*des* probe labeled at the 5′-end of either the coding or the non-coding strand was incubated with DesR-P prior to the addition of the reagents of OH˙. The mixture was subjected to a non-denaturing 5% polyacrylamide gel. The bands corresponding to free DNA, CI and CII complexes were isolated and subjected to electrophoresis in denaturing 8% polyacrylamide gels. Maxam and Gilbert (G + A) sequencing reactions were loaded in the first well. *B* shows a schematic diagram of the P*des* DNA backbone regions (*bold letters*) protected by bound DesR-P, as determined from HO˙ footprinting. *RA* and *RB* show the locations of two separate DesR-P binding regions.

was mutated to 5′-TCTAGA-3′, formed only a fast migrating complex with DesR-P. At the highest concentration of protein tested we were unable to detect full retardation of the P*desDR* promoter (Fig. 4*C*). The results of these experiments strongly suggest that DesR-P binds primarily to region A and that occupancy of this region allows DesR-P to bind to region B. As expected, P*des*D22, in which sites IR-L, IR-R, and DR were deleted, was unable to form any complex at every tested concentration (Fig. 4*C*).

To determine whether IR-L, IR-R, and DR are essential for *des* transcriptional regulation *in vivo*, P*desIR-L*, P*desIR-R*, and P*desDR* were fused to the *lacZ* reporter gene, and then integrated at the *amyE* locus of the *B. subtilis* chromosome. Under cold-shock conditions these promoter mutants showed minimal P*des* transcriptional activity (Fig. 4*B*), indicating the essentiality of IR-L, IR-R, and DR for the expression of the desaturase gene.

Cooperative Binding of DesR-P to the Pdes Binding Regions—The DNA fragments used in the above experiments contained two DesR-P binding regions, RA and RB. The fact that RB is not capable to bind DesR *per se*, suggests that cooperative interactions should play an important role in the binding of DesR-P to this site. To examine if DesR-P binds cooperatively to the two adjacent binding regions of P*des*, the levels of CI, CII, and free DNA at different concentrations of DesR-P were quantified by the PhosphorImager technology in three sets of experiments. Supplemental Materials Fig. 5 represents one of the experiments that show that the amount of DNA in CI increases first, but never exceeds 22%. In contrast, CII is present at a higher DesR-P concentration, where it becomes rapidly the predominant form. These binding characteristics suggest that DesR-P binds cooperatively to the two DesR-P binding regions. Using the data presented in Fig. 1*B*, and the equations shown under "Materials and Methods," we determined that the relative dissociation constant of CII is 12-fold less than that of CI. Thus, a DesR-P dimer binds with higher affinity RB when the neighboring region RA is occupied with another dimeric form of DesR-P.

DesR-P Activates Transcription by Stabilizing RNAP Binding at the des Promoter—One of the main mechanisms by which transcriptional activators stimulate transcription is by enhancing the RNAP binding to the promoter under control (27–28). To determine whether DesR also stimulates transcription by recruiting the RNAP, a DNA fragment extending between positions −177 and +43 was incubated with DesR-P in the absence (Fig. 5*A*, *lane 2*) or presence of *E. coli* RNAP (Fig. 5*A*, *lane 3*). Addition of RNAP leads to the formation of a heparin-resistant ternary complex that retains most of the DNA radioactivity. In the absence of DesR-P, RNAP binds poorly to P*des* (*lane 4*), generating unstable binary complexes, which are destroyed by a brief incubation with heparin (*lane 5*). As expected, no ternary complex was formed when DesRD54A was used instead DesR-P (data not shown).

To examine the transcriptional activation capacity of DesR-P on P*des*, a run-off transcription assay was performed. As shown in Fig. 5*B*, a main run-off transcript of the expected size (43 nucleotides) was produced when the template DNA was incubated with DesR-P and then with RNAP before the addition of heparin (*lane 1*), whereas no transcript was observed in the absence of DesR-P (*lane 2*). These results indicate that the heparin-resistant *Pdes*·DesR-P·RNAP ternary open complex is competent to produce run-off transcripts. It should be mentioned that in the absence of heparin, the ternary complex gives rise to a large number of nonspecific RNAs (data not shown).

DISCUSSION

Mesophile bacteria, like *B. subtilis* and *E. coli*, respond to an abrupt temperature downshift with an adaptive response that requires global changes in the gene expression pattern. Studies of this adaptive response have shown that expression of most cold-shock proteins is regulated at the post-transcriptional and translational level (29, 30). Although cold-induced activation of some promoters has been implicated in up-regulating some cold-shock genes (7, 29–31), only CspA, one of the main cold-shock proteins in *E. coli* (32, 33), and DesR (6, this study), have been identified as transcription factors specifically involved in the cold-shock response. However, the mechanism by which these transcription factors are activated at low temperature is very different. Whereas CspA mRNA is dramatically stabilized after cold shock (34), our data directly demonstrate here that DesR is activated by phosphorylation. Furthermore, the mechanism by which DesR regulates *des* transcription has been revealed here by a combination of several approaches designed to characterize the role of phosphorylation of DesR on DNA binding activity, oligomerization, and interaction with other

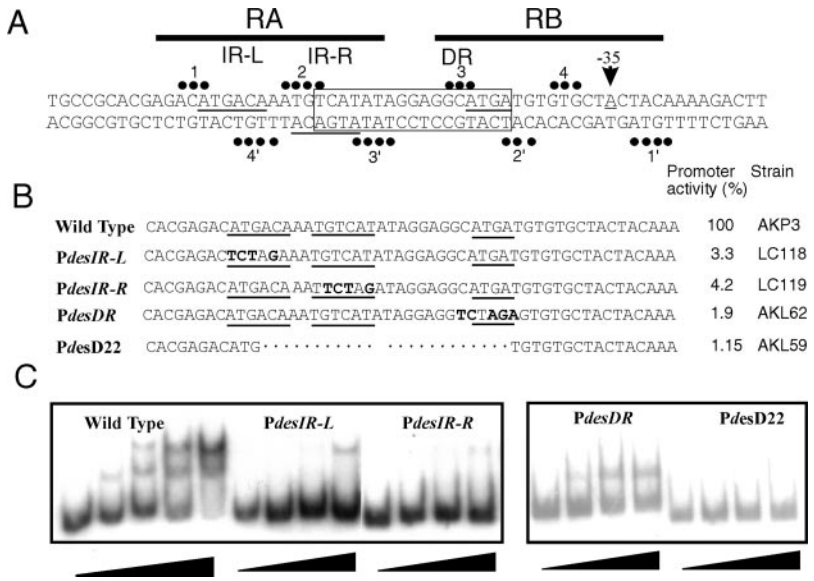

FIG. 4. *A*, *des* regulatory region. The *lines RA* and *RB* at the *top* show the location of the two DesR-P binding regions. Inverted and direct repeats are *underlined*. *Black circles* indicate bases whose deoxyriboses are protected by DesR-P from the hydroxyl radical attack. *Boxed* is the region that includes the 4-bp inverted repeat spaced 9 bp at the center of the DNase I-protected region, and the −35 site is indicated with an *arrow*. *B*, β-galactosidase activity of *des* promoter variants. The sequence changes in the promoter variants are depicted along the protected region of the *des* promoter. The nucleotide changes to introduce mutations in the inverted repeats are shown in *bold characters* and the deleted region is indicated by *dots*. *Underlined* are the positions that correspond to IR-R, IR-L, and DR. The strains were grown at 37 °C to an optical density of 0.30 and then subjected to a downshift to 25 °C. After 3 h of growth at 25 °C, the cells were harvested and β-galactosidase activities were determined. The average value of β-galactosidase activity of strain AKP3 (bearing the wild type promoter) was taken as 100% of promoter activity. The results shown are the average of three independent experiments. *C*, gel shift assay showing the binding of DesR-P (0.5 to 2 μM) to P*des* mutants. The sequence changes in the promoter variants are depicted in *B*. The assays were performed as described in the legend of Fig. 1.

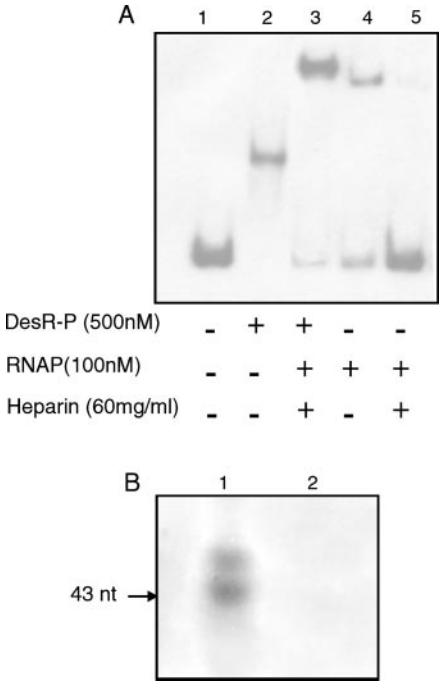

FIG. 5. *A*, analysis of the complex DesR-P·P*des*·RNAP by gel retardation. The 220-bp radiolabeled P*des* probe extending from position −177 to +43 was incubated at 25 °C with or without DesR-P and then with RNAP. Complexes were separated on non-denaturing gels. *B*, DesR-P activates transcription from P*des* templates. The 220-bp DNA fragment carrying the *des* promoter was incubated for 20 min with or without DesR-P, and then with RNAP. The mixture was treated with heparin, and the reaction was started with the addition of ribonucleotides containing [α-^{32}P]UTP. The products were analyzed by electrophoresis on an 8% polyacrylamide gel containing 8 M urea. *nt*, nucleotide.

partners in the transcription complex.

Previous studies have suggested that the unphosphorylated form of DesR was able to bind to P*des* (6). However, we found here that this binding activity was because of nonspecific phosphorylation of the response regulator during its overproduction in *E. coli*. On the basis of studying the effects of autodephosphorylation and Ac-P-mediated phosphorylation of the response regulator, we have demonstrated that the unphosphorylated form of DesR does not bind to its target DNA, and that the level of phosphorylation of DesR is proportional to its binding activity. This finding matches well with the inability of DesRD54A to bind to P*des* and to stimulate *des* transcription *in vivo*.

Sedimentation equilibrium and light scattering experiments clearly support the conclusion that DesR is a homodimer in solution. Plots of molecular mass *versus* concentration, obtained by sedimentation analysis, indicate that at low concentrations, DesR-P behaves as a dimer, but when the concentration is increased, DesR-P behaves mainly as a tetramer. These findings, together with the existence of two different DesR-P-DNA complexes strongly suggest that the phosphorylated homodimer is associated to the faster migrating complex CI, whereas the tetrameric form of DesR-P would be associated to the slower migrating complex CII. Moreover, we have determined that the binding to RA is crucial for the binding to RB (Fig. 4*C*), indicating that phosphorylation of DesR dimers is the primary recognition signal to trigger the transcriptional activation of *des*.

How could phosphorylation of DesR dimers allow target recognition? DesR belongs to the NarL group of response regulators with a phosphorylatable N-terminal regulatory domain and a C-terminal domain involved in DNA binding and transcription activation (2). The activation mechanism of NarL involves relief of inhibition. The regulatory domain obstructs the access of the recognition helix of NarL to DNA, implying that activation involves repositioning of the two domains (35). Recent data provided structural evidence that phosphorylation of the N-terminal domain of NarL triggers a global conformational change within the domain that weakens interactions at the interdomain interface. This in turn results in a separation

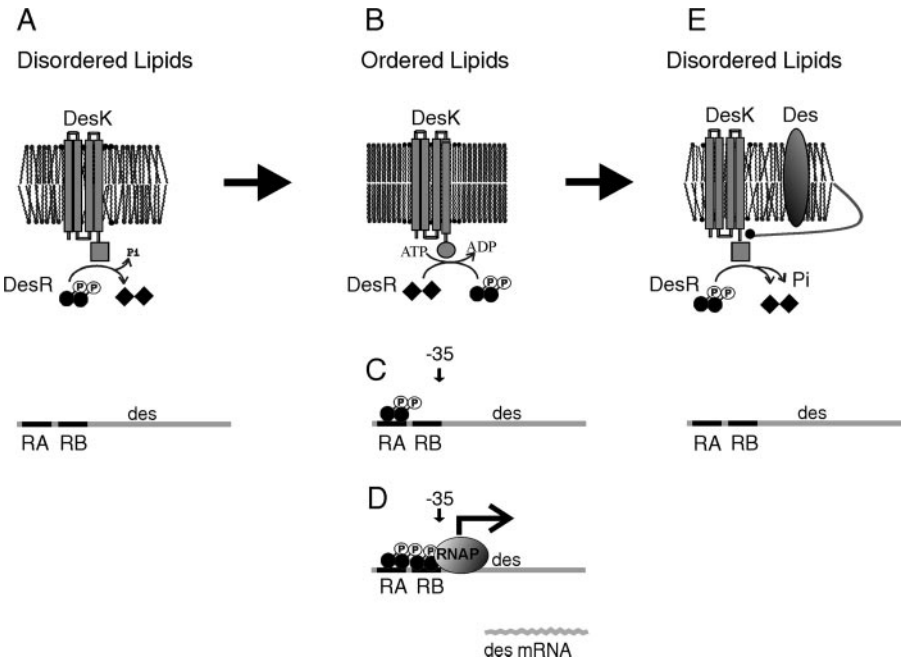

FIG. 6. **Model of *des* transcriptional control by DesK-DesR.** It is proposed that DesK assumes different signaling states in response to changes in membrane fluidity. A phosphatase-dominant state is present at 37 °C when membrane lipids are disordered (*A*). A kinase-dominant state predominates upon an increase in the proportion of ordered membrane lipids after a temperature downshift to 25 °C (6), or at 37 °C when cells were grown in minimal medium (12). *B*, a dimer of DesR receives the phosphoryl group from DesK-P, thus allowing the response regulator to interact with RA (*C*). When the levels of DesR-P increase, cooperative binding to RB results in the formation of CII, which would be essential for productive contacts with RNAP to activate *des* transcription (*D*). Activation of *des* results in synthesis of Δ5-Des, which desaturates the acyl chains of membrane phospholipids (*E*). These newly synthesized unsaturated fatty acids inhibit *des* transcription by favoring DesK dephosphorylation of DesR-P.

of the two domains exposing critical DNA binding elements in the C-terminal domain (36). By analogy with the NarL activation model, we can speculate that unphosphorylated DesR dimerizes in solution by its C-terminal domain, whereas both N-terminal regulatory domains act as inhibitors of DNA binding by steric hindrance. Upon phosphorylation, a conformational change on the N-terminal domains would occur that exposes previously inaccessible DNA-binding residues. At the same time, this movement of the N-terminal domains would expose a new protein interacting surface, supposedly in the C-terminal domain, allowing its cooperative binding to DNA regions A and B.

We have shown that at low levels of DesR-P only CI is observed resulting in protection of binding site A. In this region we found two antiparallel 6-base pair sites, with a two-nucleotide spacer between the sites, which are essential for DesR-P binding. Thus, we suggest that the dimeric form of DesR-P binds first in an antiparallel fashion to the palindromic tail-to-tail arrangement of RA of P*des*. When the levels of phosphorylated dimeric DesR are increased, cooperative binding to a second, non-palindromic site, RB, results in the formation of CII, which becomes the predominant DesR-P·DNA complex, and which would be essential for productive contacts with RNAP. From our current *in vitro* experiments, we could predict that CI will be established at about 10-fold lower concentration of DesR-P than that required for the formation of CII (Fig. 1). Because we have determined that occupancy of region B by DesR-P is crucial for transcriptional activation of P*des* (Fig. 4), it is possible that other cellular factors could decrease the concentration of DesR-P needed for the formation of the CII tetrameric complex *in vivo*. Thus, it is tempting to speculate that cooperative binding of DesR-P molecules to the regulatory region of P*des* may be an underlying principle for activation of *des* transcription. This proposal is consistent with the results of $OH^{\cdot}$ footprinting experiments that revealed: (i) occupancy of site A in complex CI, and (ii) occupancy of both sites RA and RB in complex CII, likely resulting from cooperative binding of an additional DesR-P dimer to the empty site B of CI. It should be noted that each of these sites (RA or RB) is long enough to accommodate a dimer of any helix-turn-helix DNA-binding motif protein, as it comprises two successive regions of the major groove on the same face of the DNA helix (Fig. 3*B*). In addition, our results suggest that phosphorylation of DesR has two major roles in transcriptional regulation of the desaturase gene: (i) it enables binding of DesR-P dimers to region A and (ii) it promotes cooperative interactions between DesR-P dimers at adjacent sites RA and RB that lead to tetramerization of the protein.

When RNAP first recognizes the promoter, it forms an unstable closed complex. Formation of the closed complex is fully reversible: it easily dissociates back to free RNAP and DNA, and is therefore highly sensitive to DNA competitors, such as heparin, which bind to free RNAP and prevent rebinding of the polymerase to promoter DNA (37). If RNAP commits itself to the template, the closed complex undergoes a series of conformational changes that lead to the formation of a transcription-competent open complex, which is essentially irreversible and fully resistant to DNA competitors. The fact that heparin does not destabilize the P*des*·DesR-P·RNAP ternary complex (Fig. 5*A*) indicates that a stable RNAP-promoter complex was only formed in the presence of the response regulator. These results suggest that DesR-P efficiently stabilizes the interaction between RNAP and P*des*, leading to the formation of a ternary open complex. In addition, synthesis of run-off transcripts by this complex demonstrates that no other factors are needed for the maintenance of the open complex or the promoter clearance.

A model that accounts for our present knowledge on the DesR-mediated regulatory pathway is presented in Fig. 6. Induction of the Des pathway, under a temperature downshift or by limiting the synthesis of low-melting point fatty acids, is brought about by the ability of DesK to sense a decrease in membrane fluidity (12, 13). DesK is a bifunctional enzyme with both kinase and phosphatase activities that could assume different signaling states in response to changes in membrane fluidity (12, 13). This could be accomplished by regulating the ratio of kinase to phosphatase activities, such that a phosphatase-dominant state would be present when membrane lipids are disordered resulting in dephosphorylation of the cognate response regulator DesR (A). The unphosphorylated regulator is unable to bind to P*des* and, as a consequence, *des* transcription is turned off. Upon an increase in the proportion of ordered membrane lipids, a kinase-dominant state of DesK is favored (B), so that the sensor protein undergoes autophospho-

rylation and subsequently the phosphoryl group is transferred to the cytoplasmic response regulator DesR. At low levels of phosphorylation, that is, at a low concentration of active DesR, DesR-P binds to the 6-bp palindromic sequence centered 63 bp upstream from the transcription start point of P*des* (C). However, a DesR-P dimer bound to site A would be unable to establish contacts with RNAP, thus being incompetent to activate *des* transcription. When the DesK-mediated phosphorylation of DesR increases, cooperative binding to RB is promoted (D). Tetrameric DesR-P would occupy a site centered 52 bp upstream from the transcription start point of P*des*, the dimer bound to site B being centered at position −41. This allows DesR-P to interact specifically with RNAP. Such a protein-protein contact itself would be sufficient to activate *des* transcription, as demonstrated by *in vitro* transcription experiments. Activation of *des* results in the synthesis of Δ5-Des, which introduces double bonds in the acyl chains of membrane lipids (E). These newly synthesized unsaturated fatty acids decrease the phase transition temperature of the phospholipids and inhibit the transcriptional activity of P*des*, presumably by favoring the phosphatase activity of DesK. This results in hydrolysis of DesR-P and shut-off of *des* transcription. Thus, our model argues that the regulation of *des* transcription is a direct consequence of the levels of DesR-P in the cell.

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