Reversible ADP-ribosylation is demonstrated to be a regulatory mechanism in prokaryotes by heterologous expression

(nitrogen fixation/ NH_4^+ -switch-off/*draT*/*draG*/posttranslational regulation)

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ABSTRACT The primary product of biological nitrogen fixation, ammonia, reversibly regulates nitrogenase activity in a variety of diazotrophs by a process called “ NH_4^+ -switch-off/on.” Strong correlative evidence from work in *Azospirillum lipoferum* and *Rhodospirillum rubrum* indicates that this regulation involves both the inactivation of dinitrogenase reductase by dinitrogenase reductase ADP-ribosyltransferase and the reactivation by dinitrogenase reductase activating glycohydrolase. The genes encoding these two enzymes, *draT* and *draG*, have been cloned from these two organisms, so that direct genetic evidence can be marshaled to test this model *in vivo*. The *draT/G* system has been transferred to and monitored in the enteric nitrogen-fixing bacterium *Klebsiella pneumoniae*, an organism normally devoid of such a regulatory mechanism. The expressed *draT* and *draG* genes allowed *K. pneumoniae* to respond to NH_4Cl with a reversible regulation of nitrogenase activity that was correlated with the reversible ADP-ribosylation of dinitrogenase reductase *in vivo*. Thus, the expression of *draT* and *draG* genes in *K. pneumoniae* is necessary and sufficient to support NH_4^+ -switch-off/on, and ADP-ribosylation serves as a reversible regulatory mechanism for controlling nitrogenase activity in prokaryotes.

The conversion of atmospheric nitrogen (N_2) to ammonia (NH_3) is catalyzed by the nitrogenase complex in a variety of free-living and symbiotic microorganisms (1). Nitrogenase consists of two electron-transferring proteins: dinitrogenase (MoFe protein; reduced ferredoxin:dinitrogen oxidoreductase ATP hydrolysing, EC 1.18.61) and dinitrogenase reductase (Fe protein; ferredoxin: H^+ oxidoreductase, EC 1.18.99.1). Dinitrogenase reductase transfers a single electron at a time to dinitrogenase, coupled with the hydrolysis of two molecules of ATP. The reduced dinitrogenase then transfers electrons to the substrate, N_2 , or its analogs, such as acetylene. This reaction is an energy-demanding process that consumes a theoretical minimum of 16 ATP molecules per N_2 reduced, and 20–30 ATP molecules are estimated to be hydrolysed for each N_2 reduced *in vivo* (2). It is, therefore, not surprising that N_2 -fixing microorganisms have evolved efficient mechanisms to control both nitrogenase synthesis and its activity.

Ammonia, the product of the nitrogenase reaction, regulates *nif* (nitrogen fixation) gene expression by means of the products of the *nifLA* operon and *ntr* (nitrogen regulation) system in *Klebsiella pneumoniae* and other N_2 -fixing bacteria (3). In addition, many N_2 -fixing microorganisms can post-translationally regulate the activity of the accumulated nitrogenase rapidly and reversibly in response to the availability of fixed nitrogen by a phenomenon termed “ NH_4^+ -switch-off/on” (refs. 4–7 and the references therein).

The discovery of a mono-ADP-ribosylation system for dinitrogenase reductase in the purple nonsulfur bacterium *Rhodospirillum rubrum* provided an attractive model for NH_4^+ -switch-off/on (8–10) because Kanemoto and Ludden (11) were able to correlate the loss of whole-cell nitrogenase activity upon ammonia addition with the modification of dinitrogenase reductase *in vivo*. Dinitrogenase reductase consists of two identical subunits. In *R. rubrum*, as well as the related bacterium *Rhodobacter capsulatus* (12), the attachment of a single ADP-ribose group to one subunit of the dimeric dinitrogenase reductase inactivates the enzyme system (9, 10) by disrupting the electron flow between dinitrogenase reductase and dinitrogenase (13, 14). This modification is catalyzed by dinitrogenase reductase ADP-ribosyltransferase (DRAT) with NAD serving as the ADP-ribose donor (9, 10, 12, 15). Dinitrogenase reductase activating glycohydrolase (DRAG) catalyzes the reverse reaction by removing the ADP-ribose group (8, 16, 17).

Azospirillum lipoferum, an N_2 -fixing bacterium associated in soil with the roots of nonleguminous plants, also possesses a well-studied NH_4^+ -switch-off/on system, and good evidence exists for the operation of the ADP-ribosylation system in this organism (18–22). Because the genes encoding DRAT (*draT*) and DRAG (*draG*) have been cloned from both *A. lipoferum* (22) and *R. rubrum* (23, 24), it is possible to test the model of reversible ADP-ribosylation as the molecular basis for NH_4^+ -switch-off/on. Here we report that the functional expression of *draT* and *draG* genes in a heterologous host, *K. pneumoniae*, is necessary and sufficient for the reversible regulation of nitrogenase activity by NH_4Cl , thus providing direct evidence that ADP-ribosylation of proteins serves as a reversible regulatory mechanism in prokaryotes.

MATERIALS AND METHODS

Organisms and Growth Conditions. *K. pneumoniae* strains UN5350 and UN5366 were described (24), and UN5350, carrying *F' lacI^Q*, was used as the host for the heterologous expression of *draT* and *draG* genes. *K. pneumoniae* strains were grown in LC medium (1% tryptone/0.5% yeast extract/0.5% NaCl) at 30°C. The expression of *draT* and *draG* was induced with 1 mM isopropyl β -thiogalactopyranoside (IPTG) for 4 hr or as otherwise indicated. Carbenicillin (800 $\mu\text{g/ml}$) and ampicillin (60 $\mu\text{g/ml}$) were used in selective media for *K. pneumoniae*. For *nif*-derepression of *K. pneumoniae*, an LC-grown overnight culture was inoculated (1:100) into minimal medium with ammonium acetate as the

Abbreviations: DRAT, dinitrogenase reductase ADP-ribosyltransferase; DRAG, dinitrogenase reductase activating glycohydrolase; IPTG, isopropyl β -thiogalactopyranoside; MSX, methionine sulfoximine.

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nitrogen source (25) and was grown aerobically for 24 hr. The cells then were collected ($4000 \times g$, 5 min at 4°C), resuspended in four volumes of minimal medium (without NH_4^+ ion) in the presence of serine (0.015%), and then incubated anaerobically at 30°C for 4 hr.

Expression Vector Construction. The methods of Maniatis *et al.* (26) were followed for DNA isolation, restriction enzyme digestion, ligation, and bacterial transformation.

The construction of the *A. lipoferrum* *draT/G* expression vector pHAF210 was described (22). To obtain the *draG* vector (Fig. 1), the "kanamycin cassette" of pUC4K (27) was removed at the *Sal* I sites and inserted into pHAF102, replacing the 0.9-kilobase (kb) *Sal* I fragment, producing pHAF102M8, and providing a *Pst* I site next to the *Sal* I site. The 2.1-kb *Pst* I–*Eco*RI fragment of pHAF102M8, containing *draG*, was then cloned into pKK223-3 (Pharmacia), creating pHAF211. The *draT* expression vector (pHAF213) was obtained by deleting the 0.7-kb *Bgl* II–*Eco*RI fragment of pHAF210 and replacing it with the 1.3-kb *Bgl* II–*Hind*III fragment of pHP45 Ω -Km (28).

To construct the *R. rubrum* expression vector (Fig. 4), the 2.9-kb *Bam*HI–*Bgl* II fragment of pWPF102 (23), containing the *draT/G* sequence of *R. rubrum*, was cloned into the *Bam*HI site of pUC18 (29), generating pWPF115. The downstream region of *draT/G* in this 2.9-kb *Bam*HI–*Bgl* II fragment was removed by exonuclease III digestion (Promega), and the resulting 2.0-kb fragment, containing only the *draT/G* coding region [including ≈ 200 base pairs (bp) of a noncoding sequence downstream of *draG*], was cloned into pKK223-3 (at the *Sma* I–*Hind*III sites), generating pHAF308.

Crude Extract Preparation. For assay of DRAT, *K. pneumoniae* cultures (200 ml) were grown either aerobically in LC medium or anaerobically in derepression medium, induced with IPTG for 4 hr, and collected by centrifugation at $6000 \times g$ for 15 min. The pellet was resuspended in 1.5 ml of Mops buffer (100 mM, pH 7.5, containing 1 mM ADP, 50 μM EDTA, and 1 mM dithiothreitol) and lysed in the presence of lysozyme (1 mg), DNase (1.5 mg), and RNase (1.5 mg) at 4°C for 30 min. The cells were further disrupted by sonication as described (19). After centrifugation at $125,000 \times g$ for 2 hr at 4°C , the supernatant was used as the crude extract.

For the assay of DRAG, all procedures were performed strictly anaerobically under an N_2 atmosphere. Anaerobically grown *K. pneumoniae* cells were collected by centrifugation, and the pellet was resuspended in Tris acetate buffer (250 mM, pH 7.8, containing 4 mM dithionite and 1 mM dithio-

threitol). After sonication and centrifugation as described above, the supernatant and the resuspended membrane fractions (in 25 mM Tris acetate, pH 7.8) were used for the assay.

To monitor the subunit pattern of dinitrogenase reductase, 5-ml aliquots of cultures were quickly collected on glass microfiber filters (Whatman GF/C), and the filters were immediately frozen in liquid nitrogen to quench cellular metabolism (11). Cells were disrupted by grinding the frozen filters with carborundum in an anaerobic buffer (11). After centrifugation at $8700 \times g$ for 30 sec at 30°C , samples of the supernatants were used for SDS/PAGE and immunoblotting.

Nitrogenase Assay *in Vivo*. Nitrogenase activity was measured as the acetylene reduction rate (30). To follow whole-cell nitrogenase activity, 2 ml (or 5 ml) of N_2 -fixing cultures were transferred to 25-ml serum vials that were evacuated and flushed with argon. The assay was started by injection of 2.3 ml of acetylene and then incubated at 30°C with vigorous shaking. The ethylene produced was measured with a gas chromatography unit equipped with a flame ionization detector (model GC-8A; Shimadzu, Columbia, MD). The nitrogenase activity of whole cells is expressed as nmol of ethylene formed per ml of cell culture per unit time when the OD at 600 nm was normalized to 1.0.

DRAT Assay. The procedure of Lowery *et al.* (10) was followed for DRAT activity measurement. The reaction mixture contained 0.25 mM exogenous NAD, 0.6 μCi of [α - ^{32}P]NAD (1 Ci = 37 GBq), 0.25 mM ADP, 5 mM MgCl_2 , 100 μg of purified active dinitrogenase reductase, and the crude extracts in Mops buffer (100 mM, pH 7.0) in a total volume of 50 μl . Dinitrogenase reductase from either *K. pneumoniae* or *R. rubrum* was used as substrate, and both provided consistent activities. The specific incorporation of ^{32}P into dinitrogenase reductase was verified by immunoblotting and autoradiography (21).

DRAG Assay. DRAG activity was estimated by coupling the activation of the inactive dinitrogenase reductase to the acetylene reduction assay of nitrogenase activity (16).

Determination of ADP-Ribosylation Status of Dinitrogenase Reductase by Immunoblotting. A SDS/PAGE system of Laemmli (31) was used as modified by Kanemoto and Ludden (11) to provide satisfactory resolution of dinitrogenase reductase subunits in a 10% acrylamide gel. The enzyme-linked immunoblotting procedure was followed as described earlier (19). Proteins separated in SDS/PAGE were electrophoretically transferred to nitrocellulose membranes and incubated with serum raised against dinitrogenase reductase

FIG. 1. Expression vectors for *draT* and *draG* genes of *A. lipoferrum* and their properties. (A) Expression vectors. pHAF102 was the parent plasmid where a 4.5-kb *Bam*HI–*Eco*RI fragment containing *draT* and *draG* was inserted into pUC19 as described (22). Heavy lines represent *A. lipoferrum* insert DNA, and light lines represent the vector (pUC19 or pKK223-3) portion. Arrows show the orientation of transcription of respective genes. (B) Biochemical properties of the expression vectors when expressed in *K. pneumoniae*. The DRAT activity of positives was 5–11 pmol of ADP-ribose incorporated per (mg $\times$ min), and the specific incorporation of [^{32}P]ADP-ribose into dinitrogenase reductase was verified by showing a single radiolabeled band at the position of dinitrogenase reductase on the gel; the negatives showed no labeling on the gel. DRAG activity of positives was 11–19 nmol of acetylene formed per (mg $\times$ min). Whole-cell nitrogenase activity was determined by the acetylene reduction assay. The regulatory properties were determined as described in the legend for Fig. 2.

of *Azotobacter vinelandii*; cross-reacting material was visualized with horseradish peroxidase conjugated to goat anti-rabbit IgG (Bio-Rad). The intensity of the ADP-ribosylated subunit (slower migrating) and the unmodified subunit (faster migrating) of dinitrogenase reductase was obtained by scanning the bands with a Zeineh soft laser scanning densitometer (Biomed Instruments, Fullerton, CA). These data were analyzed with the laser 1-D autostepover/videophoresis xx program (Biomed Instruments) coupled to the laser densitometer, and used to estimate the percentage of the ADP-ribosylated dinitrogenase reductase according to the method of Kanemoto and Ludden (11).

Protein Assay. Protein concentrations were determined with the micro-biuret method (32) with bovine serum albumin as the standard.

RESULTS

Expression of *A. lipoferrum draT* and *draG* Genes in *K. pneumoniae* UN5367. To study the function of the *draT* and *draG* gene products, an expression vector was constructed by placing the *A. lipoferrum draT/G* region under the control of the *tac* promoter in pKK223-3, yielding plasmid pHAF210 (ref. 22; Fig. 1A). The vector, pKK223-3, is a derivative of pBR322 and carries the *tac* promoter followed by the M13mp8 polylinker and a strong transcription terminator from the *Escherichia coli rrnB* operon. For the expression experiments, the enteric N_2 -fixing bacterium *K. pneumoniae* was chosen as a host for the following reasons: (i) there is no evidence for the NH_4^+ -switch-off/on phenomenon in this organism under any condition tested (33, 34); (ii) the dinitrogenase reductase of *K. pneumoniae* is an effective substrate for *A. lipoferrum* (data not shown) and *R. rubrum* (15) DRAT *in vitro* and can be expected to serve as an endogenous substrate for any DRAT expressed *in vivo*; and (iii) the genetics and regulation of N_2 fixation is most thoroughly understood in this organism, and a variety of defined mutants affected in nitrogen metabolism are available (3, 35).

The biochemical analyses shown in Fig. 1B demonstrate that enzyme activities of both *draT* and *draG* gene products of *A. lipoferrum* are detected in the crude extracts of *K. pneumoniae* strains transformed with pHAF210. We then investigated how a *K. pneumoniae* strain (UN5367) carrying the *draT/G* system responded *in vivo* to added NH_4^+ . Fig. 2 demonstrates that nitrogenase activity of UN5367 was reversibly regulated in response to fixed nitrogen in the medium. Before NH_4Cl addition, the nitrogenase of intact cells was active, but the addition of NH_4Cl to the medium rapidly inhibited nitrogenase activity (Fig. 2A). The inhibition was reversible, and the duration of inhibition depended on the concentration of NH_4^+ added. The reversibility of inhibition was exhibited not only upon exhaustion of the added NH_4^+ , but also by blocking the ammonium assimilation pathway. Methionine sulfoximine (MSX) is an inhibitor of glutamine synthetase, the enzyme that catalyzes the formation of glutamine from ammonia and glutamate. The addition of MSX (1.0 mM) to the medium before addition of NH_4^+ prevented the NH_4^+ inhibition, and MSX reversed the inhibition when added after NH_4^+ (Fig. 2B). This result suggests that, although NH_4^+ itself is not a direct regulator, a metabolic product of NH_4^+ plays an important role. Consistent with this idea, Fig. 2C shows that glutamine, like NH_4^+ , reversibly inhibits whole-cell nitrogenase activity. In contrast, NH_4Cl or glutamine had no effect on nitrogenase activity of UN5366, which carries plasmid pKK223-3 without the *draT/G* insert (Fig. 2D).

The effects of NH_4^+ , glutamine, and MSX observed in this constructed *K. pneumoniae* system are similar to those documented in *A. lipoferrum* (19, 21). Thus, the introduction of the *draT/G* expression system into *K. pneumoniae* confers

Fig. 2. Reversible regulation of nitrogenase activity in *K. pneumoniae* UN5367 (pHAF210). After the *nif* regulon was derepressed anaerobically, whole-cell nitrogenase activity was monitored in serum vials. The assays were started by injection of acetylene, followed by incubation at 30°C with vigorous shaking. The reagents noted below were introduced into the medium at times indicated by arrows. (A) Effects of NH_4Cl on nitrogenase activity (0.2 mM, Δ ; 0.4 mM, $\triangle$; and 1.0 mM, $\blacksquare$). (B) Effect of MSX on NH_4^+ inhibition of nitrogenase activity. MSX (1.0 mM) was added either before ($\square$) or after the addition of NH_4Cl (1.0 mM) ($\triangle$) or 1.0 mM NH_4Cl alone ($\blacksquare$). (C) Effect of glutamine on nitrogenase activity at 0.4 mM (Δ) or 1.0 mM ($\blacksquare$). (D) Effect of NH_4Cl (1.0 mM, Δ) or glutamine (1.0 mM, $\triangle$) on nitrogenase activity in *K. pneumoniae* UN5366 (pKK223-3). For each case the control line reflects results obtained when H_2O was added at 30 min ($\circ$).

an NH_4^+ -switch-off/on mechanism remarkably similar to that of *A. lipoferrum*.

ADP-Ribosylation of Dinitrogenase Reductase in *K. pneumoniae* UN5367. The following experiments indicate that the dinitrogenase reductase in *K. pneumoniae* UN5367 cells carrying pHAF210 becomes ADP-ribosylated in response to NH_4^+ treatment. Dinitrogenase reductase consists of two identical subunits, only one of which becomes ADP-ribosylated upon inactivation, and the ADP-ribosylated subunit can be separated from the unmodified subunit by SDS/PAGE (11). Before addition of NH_4Cl to UN5367, no ADP-ribosylated dinitrogenase reductase was detectable (Fig. 3). After addition of NH_4Cl , whole-cell nitrogenase activity was inhibited (Fig. 2A), and the dinitrogenase reductase rapidly became ADP-ribosylated (Fig. 3). Upon recovery of nitrogenase activity, the concentration of ADP-ribosylated dinitrogenase reductase decreased dramatically, consistent with the duration of inhibition of nitrogenase activity. As controls, the dinitrogenase reductase of wild-type *K. pneumoniae* had only the unmodified form of dinitrogenase reductase with or without NH_4Cl treatment.

Separate Expression of *draT* and *draG*. Additional expression vectors were constructed to analyze the effect of the separate expression of the *draT* and *draG* genes (Fig. 1). Deletion of *draG* (pHAF213) had no effect on DRAT activity; the whole-cell nitrogenase activity in a strain (UN5370) with this plasmid is negligible under the normal derepression conditions in the presence of inducer IPTG because the dinitrogenase reductase is always ADP-ribosylated (data not

FIG. 3. Reversible ADP-ribosylation of dinitrogenase reductase in *K. pneumoniae* (UN5367) upon NH_4Cl treatment. The percentage of the ADP-ribosylated dinitrogenase reductase was monitored during the whole-cell nitrogenase assay by immunoblotting. The following strains and conditions were tested: *K. pneumoniae* UN5367 ($\text{draT}^+ \text{draG}^+$) at 0.2 mM NH_4Cl (○) and 1.0 mM NH_4Cl (□); *K. pneumoniae* UN5350 ($\text{draT}^- \text{draG}^-$) at 1.0 mM NH_4Cl (■). Arrow indicates NH_4Cl addition.

shown). Deletion of *draT* (pHAF211) did not affect DRAG activity. When pHAF211 was expressed in *K. pneumoniae* (UN5368), neither the regulation of nitrogenase activity nor modification of dinitrogenase reductase occurred upon addition of NH_4^+ . The removal of the 0.4-kb *Sal*I-*Eco*RI fragment of pHAF210 (yielding pHAF212) had no effect on DRAT or DRAG activities in *K. pneumoniae*; nitrogenase activity was regulated by NH_4Cl as in strains carrying pHAF210 (data not shown). Therefore, clearly both *draT* and *draG*, but not the region immediately downstream from them, are required for the NH_4^+ -regulatory system for nitrogenase activity.

Expression of *R. rubrum draT* and *draG* Genes in *K. pneumoniae* UN5377. Because the sequence of the *draT* and *draG* genes of *R. rubrum* had been determined (23), it was important to confirm the results obtained with *A. lipoferrum draT* and *draG* genes by construction and testing of a similar expression vector with *R. rubrum draT* and *draG* (Fig. 4A). For this purpose pHAF308 was constructed, which contains only the *draT* and *draG* coding region of *R. rubrum*. Fig. 4B demonstrates that NH_4Cl reversibly regulated nitrogenase activity in a *K. pneumoniae* strain carrying pHAF308. The inhibition is correlated with the presence of ADP-ribosylated dinitrogenase reductase (data not shown).

DISCUSSION

The data in this report demonstrate that the expression of cloned *draT* and *draG* genes of two unrelated diazotrophs is necessary and sufficient to confer the NH_4^+ -switch-off/on capability on *K. pneumoniae* and that the dinitrogenase reductase in these cells is reversibly ADP-ribosylated in response to NH_4^+ treatment. Since the discovery of mono-ADP-ribosylation as the mechanism of action of diphtherial toxin (37), ADP-ribosylation has been suggested to play a role in normal cell metabolism by regulating the activity of key enzymes. This study provides direct genetic evidence that ADP-ribosylation serves as a reversible regulatory mecha-

A Construction of expression vector

B

FIG. 4. Reversible regulation of nitrogenase activity in *K. pneumoniae* strain carrying *R. rubrum draT* and *draG*. (A) Vectors for *draT* and *draG* of *R. rubrum*. Heavy lines represent *R. rubrum* insert DNA, and light lines represent the vector portion. (B) Effect of NH_4Cl addition on whole-cell nitrogenase activity. The experiments were done as described in the legend to Fig. 2, except that IPTG was omitted because the uninduced expression of *draT* and *draG* was sufficient for effect (22, 24, 36). At 30 min, NH_4Cl was injected to give final concentrations of 0.0 mM (○), 0.4 mM (■), 1.0 mM (□), and 10 mM (▲).

nism for cellular metabolism in prokaryotes by controlling nitrogenase activity.

The expression of *draT* and *draG* in *K. pneumoniae* has conclusively established the *in vivo* function of DRAT and DRAG and raises questions about the nature of the decision-making mechanism of this regulatory system. The expression of *draT* alone produced a Nif^- phenotype in *K. pneumoniae* because dinitrogenase reductase was ADP-ribosylated, regardless of the level of exogenous NH_4^+ . Although the expression of *draG* alone had no effect on nitrogenase activity with or without NH_4^+ treatment, its expression was necessary for the reversible regulation of nitrogenase activity *in vivo* in response to exogenous reduced nitrogen compounds. These observations suggest that DRAG activity (or both DRAG and DRAT) is regulated posttranslationally in response to the availability of reduced nitrogen. This regulation causes a decrease in nitrogenase activity with the rise in the level of NH_4^+ (DRAT activity dominant) and supports the recovery of activity upon the depletion of the reduced NH_4^+ (DRAG activity dominant). Evidence for the regulation of DRAG activity in *R. rubrum* has been obtained by following the rate of ADP-ribose turnover during NH_4^+ inhibition (11). After addition of NH_4^+ to a culture, the turnover rate for [^{32}P]ADP-ribose attached on dinitrogenase reductase was much slower than that for cellular phosphate, indicating that DRAG was not active *in vivo* under these conditions.

Nitrogenase activity in wild-type *K. pneumoniae* is not sensitive to NH_4^+ under normal derepression conditions. Results reported here indicate that the expression of DRAT and DRAG activities is sufficient for reversible regulation of nitrogenase activity by NH_4^+ . Because no supplementary

genetic information was necessary for appropriate functioning of DRAT and DRAG in *K. pneumoniae*, this organism must contain sufficient ancillary functions for nitrogenase regulation when provided with *draT* and *draG* from *A. lipoferum* or *R. rubrum*. This result suggests that the signal for NH_4^+ regulation of the DRAT/DRAG system is present in an organism that normally does not carry out ADP-ribosylation of nitrogenase. The functional expression of *draT* and *draG* genes in the extensively studied *K. pneumoniae* system will facilitate the elucidation of this signal-transduction pathway leading to the ADP-ribosylation of dinitrogenase reductase.

This work shows that apparently complete posttranslational regulatory machinery for nitrogenase activity can be transferred between diverse organisms and operate functionally there. This result may have evolutionary implications for the cell's mechanism of obtaining new functions or even complete regulatory machinery through gene transfer.

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