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Terminal Oxidases Are Essential To Bypass the Requirement for ResD for Full Pho Induction in *Bacillus subtilis*

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The *Bacillus subtilis* Pho signal transduction network, which regulates the cellular response to phosphate starvation, integrates the activity of three signal transduction systems to regulate the level of the Pho response. This signal transduction network includes a positive feedback loop between the PhoP/PhoR and ResD/ResE two-component systems. Within this network, ResD is responsible for 80% of the Pho response. To date, the role of ResD in the generation of the Pho response has not been understood. Expression of two terminal oxidases requires ResD function, and expression of at least one terminal oxidase is needed for the wild-type Pho response. Previously, our investigators have shown that strains bearing mutations in *resD* are impaired for growth and acquire secondary mutations which compensate for the loss of the *a*-type terminal oxidases by allowing production of cytochrome *bd*. We report here that the expression of cytochrome *bd* in a Δ*resDE* background is sufficient to compensate for the loss of ResD for full Pho induction. A *ctaA* mutant strain, deficient in the production of heme A, has the same Pho induction phenotype as a Δ*resDE* strain. This demonstrates that the production of *a*-type terminal oxidases is the basis for the role of ResD in Pho induction. Terminal oxidases affect the redox state of the quinone pool. Reduced quinones inhibit PhoR autophosphorylation in vitro, consistent with a requirement for terminal oxidases for full Pho induction in vivo.

The *Bacillus subtilis* phosphate starvation response (Pho response) is under the control of a complex regulatory network that allows the cell to respond to the level of inorganic phosphate (P_i) in the environment. This system is critical to survival because phosphate is the limiting nutrient in soil (33), the natural environment for *B. subtilis*.

Central to the *B. subtilis* Pho response is the PhoP/PhoR two-component signal transduction system. The *phoPR* operon (23, 43) is subject to activation by PhoP under phosphate starvation conditions (34). PhoP/PhoR directly regulates the expression of genes involved in the cellular response to phosphate starvation. The histidine kinase, PhoR, is autophosphorylated in response to an environmental signal and then phosphorylates its cognate response regulator, PhoP. PhoP∼P activates the transcription of the alkaline phosphatases (19), *phoA* (formerly *phoAIV*) (20), and *phoB* (formerly *phoAIII*) (7); phosphodiesterases, *phoD* (11), and *glpQ* (1); a high-affinity phosphate transport system, *pstS* (37); teichuronic acid synthetic genes (teichuronic acid is a cell wall polymer lacking phosphate), *tuaABCDEFGH* (27, 46); and a gene encoding a 60-residue peptide of unknown function, *ykoL* (38). PhoP∼P has been shown to repress the expression of the *tagAB* and *tagDEF* genes responsible for the production of teichoic acid (a cell wall polymer containing phosphate) (26). The collective action of these products allows the cell to scavenge extracellular phosphate and to release additional P_i from the cell wall.

The regulation of the Pho response integrates the activity of three two-component signal transduction systems into a regulatory network. These systems are the PhoP/PhoR two-component system, the ResD/ResE two-component system, and the sporulation initiation phospho-relay (6). Previous work (49) has shown that both ResD and AbrB are required for full Pho induction. A Δ*resDE* strain lacks 80% of the wild-type Pho response, while a Δ*abrB* strain lacks 20% of the wild-type Pho response. A *resD abrB* double mutant shows no Pho induction, the same phenotype as a *phoPR* mutant strain (20), demonstrating that both ResD and AbrB are required upstream of PhoP and PhoR for full Pho induction. A *spoOA* mutation in the background of either the *resD* or *abrB* mutations leads to hyperinduction of the Pho regulon genes (49). A *spoOA* mutation in the *resD abrB* double mutant background does not alter the Pho-negative phenotype, indicating that only two upstream pathways, ResD and AbrB, regulate Pho induction, each of which is negatively regulated by SpoOA (3). Previous work has demonstrated that PhoP is an essential transcriptional activator of the promoter upstream of the *resABCDE* operon under phosphate starvation conditions (3). In addition, PhoP represses transcription from a minor internal promoter upstream of *resD* (3). These data together with the fact that ResD is required for 80% of the wild-type Pho response (49) demonstrate that a feedback loop exists between the ResD/ResE system and the PhoP/PhoR system. Both ResD and PhoP activate transcription from their own promoters under phosphate starvation conditions, adding to the feedback amplification (3, 25, 34). The question addressed here is how ResD generates 80% of the wild-type Pho response.

The primary role of the ResD/ResE two-component system is in the cellular response to respiratory conditions. ResD activates genes involved in both aerobic and anaerobic respi-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
E. coli strain		
DH5α		Lab stock
B. subtilis strains		
JH12586	*trpC2* Δ*abrB*::Cm^r	J. A. Hoch
JH642	*pheA1 trpC2*	J. A. Hoch
MH5124	*pheA1 trpC2 phoR*Δ*Bal*I::Tet^r	44
MH5202	*pheA1 trpC2*Δ*resDE*::Tet^r	50
MH5857	*pheA1 trpC2*Δ*resDE*::Tet^r *ydiH amyE*::*phoD-lacZ Cm^r*	41
MH5878	*pheA1 trpC2 amyE*::*cydA-lacZ* Cm^r	41
MH5879	*pheA1 trpC2* Δ*resDE*::Tet^r *ydiH amyE*::*cydA-lacZ* Cm^r	41
MH5880	*pheA1 trpC2* Δ*resDE*::Tet^r *amyE*::*cydA-lacZ* Cm^r	41
MH5884	*pheA1 trpC2* P_{spac}-*cydABCD* Cm^r	41
MH5885	*pheA1 trpC2* P_{spac}-*cydABCD* Cm^r Δ*resDE*::Tet^r	41
MH5887	*pheA1 trpC2*Δ*resDE*:Tet^r *ydiH*	Guofu Sun
MH5888	*pheA1 trpC2*Δ*resDE*:Tet^r *ydiH*	Ruth Chestnut
MH6303	*pheA1 trpC2 phoR*Δ*Bal1*::Tet^r *amyE*::*cydA-lacZ* Cm^r	This study
MH7124	*pheA1 trpC2 ctaA*AΩpAE25 Spc^r	This study
MH7128	*pheA1 trpcC2 ctaA*AΩpAE25 Spc^r P_{spac}-*cydABCD* Cm^r	This study
MH7131	*pheA1 trpC2*Δ*resDE*::Tet^r *ydiH* Δ*abrB*::Cm^r	This study
Plasmids		
pCR2.1	Vector for cloning PCR products	Invitrogen
pDH32	Vector for construction of promoter-*lacZ* fusions, Amp^r Cm^r	45
pDG1727	Vector for mutant generation, Spc^r	14
pDH88	Vector containing the P_{spac} promoter for creation on an IPTG-inducible promoter	16
ECE 75	Vector for antibiotic conversion, Cm^r to Tet^r	47
pMS34	Fragment of *cydA* promoter in pCR 2.1, Amp^r	41
pMS35	*cydA-lacZ* fusion in pDH32, Amp^r Cm^r	41
pMS37	Fragment of *cydA* in pCR2.1, Amp^r	41
pMS38	P_{spac}-*cydABCD* in pDH88, Amp^r Cm^r	41
pMS40	Internal fragment of *ydiH* in pCR2.1, Amp^r	41
pMS45	Internal fragment of *ydiH* in pDG1727, Amp^r Spc^r	41
pAE24	Internal fragment of *ctaA* in pCR2.1, Amp^r	This study
pAE25	Internal fragment of *ctaA* in pDG1727, Amp^r Spc^r	This study

ration, including *fnr* (32), *hmp* (32), *nasDEF* (32), *hemN* (17), *hemZ* (17), and the *sbo-alb* operon (31) under anaerobic conditions and *ctaA* (35, 56), *ctaBCDEF* (28), *resABCDE* (50), and *petCBD* (50) under aerobic conditions. ResD plays a critical role in the production of *a*-type terminal oxidases during aerobic respiration. CtaB catalyzes the conversion of heme B to heme O (28), which is then converted to heme A by CtaA (51). Strains lacking heme A cannot produce terminal oxidases aa_3 or caa_3 (30). In addition, *ctaCDEF* are the structural genes for cytochrome caa_3 (2). Because ResD is required for *ctaA* and *ctaBCDEF* transcription, a *resD* mutant lacks both *a*-type terminal oxidases (49).

Three terminal oxidases have been described in *B. subtilis*. These include two *a*-type heme-copper oxidases, cytochromes aa_3 (40) and caa_3 (10). The third terminal oxidase is a member of the cytochrome *bd* family (54). A putative fourth terminal oxidase, YthAB, has been suggested from sequence homology and is proposed to be a member of the cytochrome *bd* family (53).

Previously, we demonstrated that Δ*resDE* strains spontaneously acquire secondary mutations which bypass some Δ*resDE* phenotypes, including restoring Pho induction to wild-type levels (41). These secondary mutations are loss-of-function mutations in *ydiH*, which encodes a negative regulator of the *cydABCD* operon, which encodes cytochrome *bd* in *B. subtilis* (41). The expression of cytochrome *bd* in a Δ*resDE* background is sufficient to bypass those *resDE* mutant phenotypes that are not directly associated with the loss of ResD as a transcription activator (41). These data led to the hypothesis that the activity of the terminal oxidases is involved in the role of ResD in the generation of the Pho response as examined here.

MATERIALS AND METHODS

Strains and plasmids. Table 1 lists the strains and plasmids used in this study. *Escherichia coli* DH5α was the host for all plasmid constructions. *B. subtilis* JH642 was the host for all strain constructions. The construction of strains MH5878, MH5879, MH5880, MH5884, and MH5885 and of plasmids pMS34, pMS35, pMS37, pMS38, pMS40, and pMS45 was described previously (41). Chromosomal DNA from JH12586 (Δ*abrB*) was transformed into MH5888 (Δ*resDE ydiH*), creating MH7131. Transformation of chromosomal DNA from MH5124 (Δ*phoR*) into MH5878 created MH6303.

To create an insertion duplication mutation in *ctaA*, we cloned an internal fragment into pDG1727. Primers FMH831 (5′-AGAAGCTT^{91}TCCGGCCAAG GATGCGGCAGACAGTG^{116}-3′; HindIII site underlined) and FMH832 (5′-G GTCTAGA^{820}AGCCCAGAGCCAGTTCAGAGTATACG^{795}-3′; XbaI site underlined) were used to amplify a 729-bp internal fragment of *ctaA*, using JH642 as the template DNA. This fragment was cloned to pCR2.1 (Invitrogen) to create pAE24. The *ctaA* fragment was released by digestion with EcoRV and BamHI and cloned into the complementary sites in pDG1727 to create pAE25. pAE25 was transformed to JH642 to create MH7124 (Ω*ctaA*). The disruption of the *ctaA* gene was confirmed by PCR. The strain exhibited growth phenotypes associated with *ctaA* mutant strains on tryptose blood agar base plates (TBAB) and TBAB plates with 0.5% glucose (TBABG) (30). It also showed the phenotypes associated with a *ctaA* mutant strain with respect to acid accumulation on purification agar and sporulation (30). Due to the stress placed on the cell by the *ctaA* mutation, these strains were grown in the presence of spectinomycin to prevent loss of the plasmid insertion by recombination. To examine the effect of expressing cytochrome *bd* in a *ctaA* mutant background, the strain MH7128 (Ω*ctaA*

P_{spac}-cydABCD) was made by transformation of chromosomal DNA from MH7124 (Ω*ctaA*) into MH5884 (*P_{spac}-cydABCD*).

Genetic manipulations. Transformation of *B. subtilis* was done by the two-step transformation method of Cutting and Vander Horn (9). Transformants were selected on TBABG with the appropriate antibiotic. Antibiotics were added for selection of *B. subtilis* transformants at the following concentrations: chloramphenicol (5 μg/ml), spectinomycin (100 μg/ml), and tetracycline (10 μg/ml). Transformation of *E. coli* was done according to the method of Hanahan (15). Transformants were selected on Luria-Bertani (LB) plates containing ampicillin (100 μg/ml).

Growth conditions and enzyme assays. Total alkaline phosphatase (APase) activity (APase expression from *phoA* and *phoB*) was measured in cells that had been grown in low-phosphate defined medium (LPDM) as described previously (18) or high-phosphate defined medium (HPDM). LPDM contains 0.4 mM P_i; HPDM contains 5.0 mM P_i. One unit of APase activity was defined as 1 μM *para*-nitrophenol produced min^{-1}, and specific activity was calculated as units of APase per milliliter. β-Galactosidase activity was detected using the method of Ferrari et al. (12). The activity unit was defined as 0.33 nmol of *ortho*-nitrophenol produced min^{-1}, and the specific activity was calculated as activity per milligram of protein. When appropriate, isopropyl-β-D-thiogalactosidase (IPTG) was added at a final concentration of 1 mM throughout growth. Culture density and APase activity were measured hourly from cells grown under culture conditions described previously (7).

Preparation and spectrophotometry of solubilized membrane vesicles. Membrane vesicles were prepared as described by Bisschop and Konings (4) with the following modifications. Cells were collected from Pho-induced stationary-phase cultures grown in LPDM. DNase and RNase were omitted from the lysis procedure. Solubilization of cytochromes and analysis of difference absorption spectra were performed as described by Mueller and Taber (30). Difference absorption spectra (dithionite reduced minus ferricyanide oxidized) were recorded at room temperature at a scan speed of 5 nm/s with a Hitachi U-2000 spectrophotometer. Reduction and oxidation were performed as previously described (30).

Purification of PhoR and phosphorylation assays. Overexpression and purification of the truncated histidine kinase $*PhoR^{231–589}$ was performed as described previously (44). Purified protein $*PhoR^{231–589}$ (3 μM) was incubated with 5 μCi of [γ-^{32}P]ATP (specific activity, 6,000 ci/mmol; 10 mCi/ml; Amersham Biosciences) at room temperature in P-buffer (50 mM HEPES [pH 8.0], 50 mM KCl, and 5 mM $MgCl_2$) in the presence or absence of menadione (MK_3; 2 mM; Sigma-Aldrich) and/or dithionite (2 mM; Sigma-Aldrich). The reaction was started by the addition of [γ-^{32}P]ATP. Samples were taken at the indicated time intervals (see Fig. 6), and the reaction was terminated by adding an equal volume of 4× sodium dodecyl sulfate (SDS) loading sample buffer. Samples were subjected to SDS-polyacrylamide gel electrophoresis on 12.5% polyacrylamide gels (22). Radioactivity of the PhoR~P protein on the dried gels was detected with a PhosphorImager (Molecular Dynamics) and was quantitated by using ImageQuant (version 5.1).

RESULTS

Δ*resDE* mutant strains bearing *ydiH* mutations have wild-type levels of Pho induction. Our investigators have previously shown that *B. subtilis* Δ*resDE* strains that express cytochrome *bd* during growth in LB plus 0.5% glucose are complemented for those *resD* mutant phenotypes that are not directly associated with the role of ResD as a transcriptional activator (41). Strains bearing mutations in *resD* have approximately 20% of the wild-type Pho response during phosphate deprivation (49). We asked if the *ydiH* mutation which suppresses the Δ*resDE* phenotype is sufficient to bypass the requirement of ResD for full Pho induction. The APase specific activities in the parental strain (MH5857), the Δ*resDE* strain (MH5880), and the Δ*resDE ydiH* strain (MH5879) grown in LPDM showed that the *ydiH* mutant strain had wild-type levels of Pho induction despite the fact that *resD* is not functional (Fig. 1).

Cytochrome *bd* is expressed in a Δ*resDE ydiH* mutant strain under phosphate starvation conditions. If cytochrome *bd* were essential to Pho induction in a Δ*resDE ydiH* strain, we would expect to see aberrant expression of cytochrome *bd* under phosphate starvation conditions. Expression of a *cydA-lacZ*

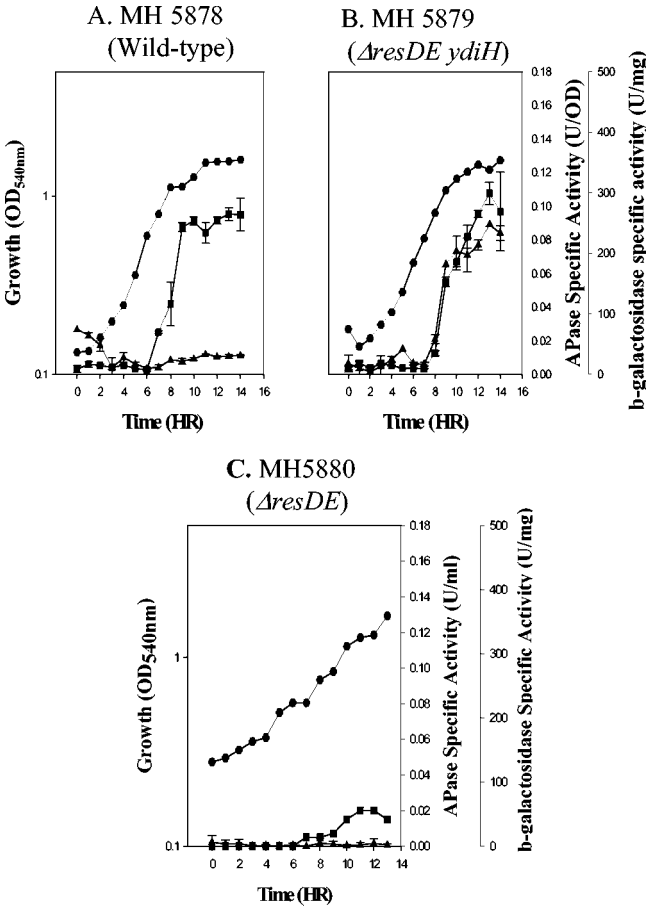

FIG. 1. *cydA-lacZ* and APase expression in strains cultured in LPDM. ●, growth; ■, APase production; ▲, *cydA-lacZ* expression. (A) MH5878 (wild-type *cydA-lacZ*); (B) MH5879 (Δ*resDE ydiH cydA-lacZ*); (C) MH5880 (Δ*resDE cydA-lacZ*).

fusion and APase induction was assayed in a wild-type background, a *ydiH* Δ*resDE* background, and a Δ*resDE* background (Fig. 1) from cells grown in LPDM. The *cydA-lacZ* fusion was expressed at low levels in the wild-type strain (Fig. 1A) and was not expressed in a Δ*resDE* strain (Fig. 1C) under phosphate starvation conditions. This suggests that cytochrome *bd* is a minor terminal oxidase under these growth conditions. In the suppressed Δ*resDE* strain bearing the *ydiH* mutation (MH5879) (Fig. 1B), the *cydA-lacZ* fusion was highly expressed during stationary phase, and APase was restored to wild-type levels. This expression was 10-fold higher than that seen in the wild-type background (MH5878) (Fig. 1A). These data suggest that elevated expression of cytochrome *bd* (and perhaps other terminal oxidases) is able to restore full Pho induction in a Δ*resDE* mutant strain.

Additional evidence for the increased expression of cytochrome *bd* in a Δ*resDE ydiH* strain was shown by the light absorption difference (dithionite-reduced minus ferricyanide-oxidized) spectra from a strain bearing the *ydiH* Δ*resDE* mutation grown to stationary phase in LPDM. Strains bearing a Δ*resDE* mutation lack *a*-type cytochromes and therefore do not produce the spectral peak at 600 nm (50) that is observed in wild-type membranes (Fig. 2A). Differential spectra of

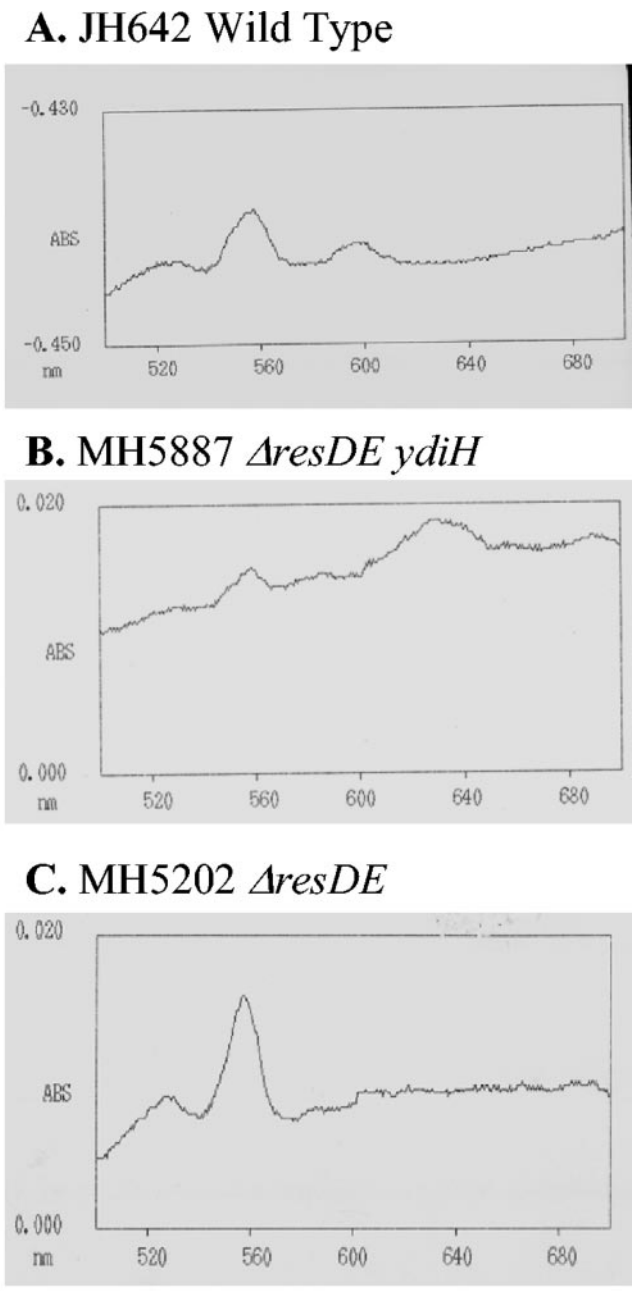

FIG. 2. Light absorption difference (dithionite-reduced minus ferricyanide-oxidized) spectra of membranes from *B. subtilis* strains. JH642 (wild type), MH5202 (Δ*resDE*), and MH5202 (Δ*resDE ydiH*) were grown in LPDM and harvested during Pho induction. A representative result is shown for each strain. (A) JH642; (B) MH5857; (C) MH5202.

membranes from a Δ*resDE ydiH* strain grown in LPDM showed the expected pattern for cytochrome *bd* expression (Fig. 2B), namely, a trough at 650 nm and peaks at 622 and 595 nm (54). The characteristic cytochrome *bd* pattern was not observed in a strain bearing a Δ*resDE* mutation (Fig. 2C) or in membranes from a wild-type strain grown under the same conditions (Fig. 2A). These data confirm that cytochrome *bd* is expressed during Pho induction in LPDM in the suppressed Δ*resDE ydiH* double mutant.

Mutations in *ctaA* mimic the Δ*resDE* Pho-defective phenotype. To test whether the loss of terminal oxidases was responsible for the role of ResD in Pho induction, we determined the level of Pho induction in a *ctaA* mutant strain. ResD is a direct transcriptional activator of *ctaA* (35, 56). A Δ*resDE* strain lacks CtaA and cannot synthesize heme A (50), leading to a deficiency in the *a*-type terminal oxidases aa_3 and caa_3. A strain bearing a *ctaA* mutation has some of the growth phenotypes associated with the loss of the *a*-type terminal oxidases that are seen in a Δ*resDE* strain (50) Compared to the parental strain, a *ctaA* mutant showed decreased Pho induction similar to that of a Δ*resDE* strain (compare Fig. 3A and B). Thus, the lack of *a*-type terminal oxidases, owing to a mutation in either *resD* or *ctaA*, resulted in decreased Pho induction. Interestingly, the *ctaA* mutant strain grew at a similar rate as the wild-type strain in LPDM (Fig. 3). Further, this suggests that the depression of Pho induction in a Δ*resDE* strain is associated with the lack of terminal oxidases, rather than the poor growth phenotype of the strain.

Expression of the *cydABCD* operon is sufficient to bypass the requirement for either ResD or CtaA for full Pho induction. To confirm that cytochrome *bd* expression is responsible for the restoration of growth and Pho induction in a *ydiH* strain, we used strain MH5885 (Δ*resDE* P_{spac}-*cydABCD*), which allowed control of the production of cytochrome *bd* in a Δ*resDE* background. If the role of ResD in Pho induction were not the result of direct transcriptional activation of the *phoPR* operon, then the expression of cytochrome *bd* might be sufficient to restore Pho induction to wild-type levels, because our group previously showed (41) that induction of cytochrome *bd* in a Δ*resDE* background was sufficient to restore other phenotypes resulting from the absence of terminal oxidase production in a *resDE* mutant.

Strain MH5885 (Δ*resDE* P_{spac}-*cydABCD*), when grown in LPDM in the presence of IPTG (Fig. 4A), showed growth and Pho induction similar to that of a Δ*resDE ydiH* strain (MH5887) (Fig. 4A), indicating that expression of cytochrome *bd* was sufficient to bypass the requirement for ResD for full Pho induction. Further, because IPTG was present at the time of inoculation of MH5885 and APase induction in that strain is coincident with APase expression in the *ydiH resDE* strain, a strain shown to induce *cydA* postexponentially (Fig. 1B), this suggests that Pho induction is not dependent on when *bd* is made, only that *bd* is there when phosphate deficiency occurs. When IPTG was absent (Fig. 4A), MH5885 (Δ*resDE* P_{spac}-*cydABCD*) showed the reduced growth rate associated with a *resD* strain, which resulted in APase specific activity reaching 0.02 after 14 h, a similar APase specific activity as expressed in the Δ*resDE* strain upon phosphate starvation (data not shown).

To determine if expression of cytochrome *bd* in a *ctaA* mutant background could restore a wild-type level of Pho induction, we combined a *ctaA* mutation with the IPTG-inducible *cydABCD* operon (MH7128). Expression of APase in MH7128 (Ω*ctaA* P_{spac}-*cydABCD*) grown in LPDM in the presence of IPTG (Fig. 4B, lane 3) showed a wild-type level of induction (JH642) (lane 1), whereas the same strain grown without IPTG (lane 4) had a low Pho induction level, similar to that of a *ctaA* mutant strain (lane 2). The fact that the *ctaA* mutant strain containing P_{spac}-*cydABCD* (MH7128) had a higher level than the parental strain (*ctaA*) is likely due to low expression from

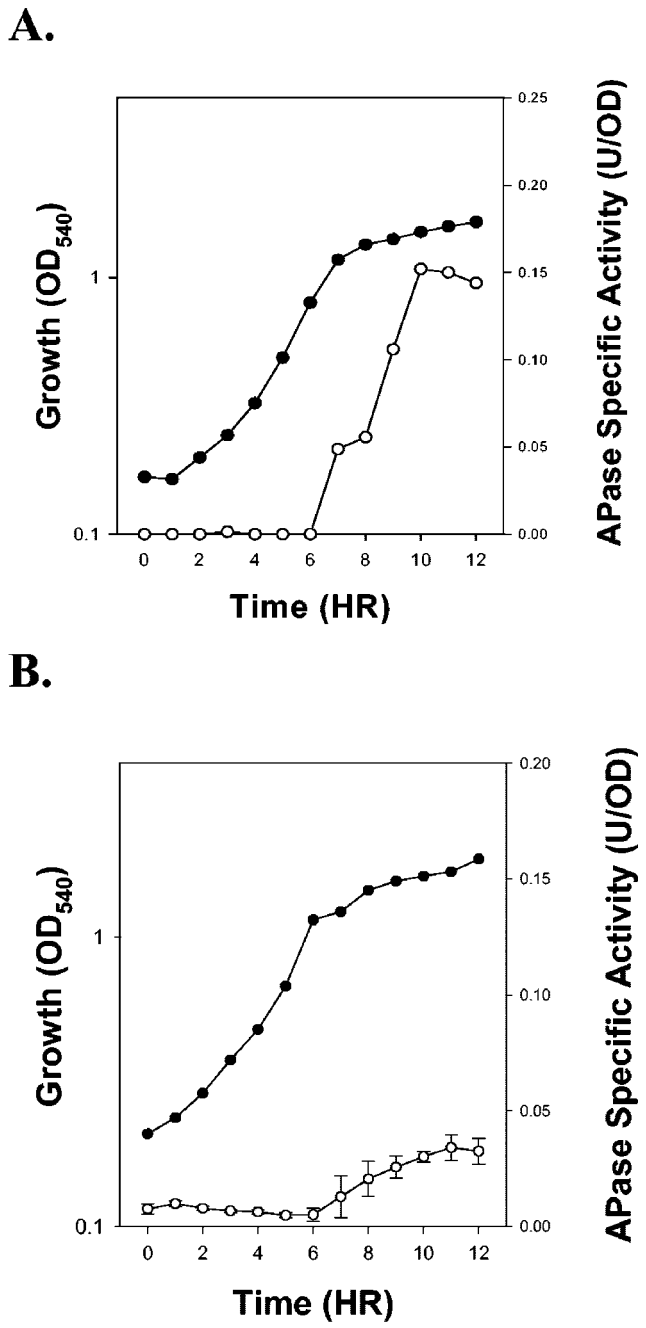

FIG. 3. Effect of *ctaA* on growth and APase production in strains cultured in LPDM. Solid symbols, growth; open symbols, APase specific activity. (A) JH642 (wild type); (B) MH7124 (Ω*ctaA*). The results for MH7124 (Ω*ctaA*) are the average of readings from five *ctaA* mutant clones.

the P_{spac} promoter in the absence of IPTG. These data, taken together with the results of similar assays in a Δ*resDE* background (Fig. 4A), confirm that expression of a terminal oxidase in either a *ctaA* or a *resD* mutant background is sufficient to restore Pho induction to wild-type levels. This demonstrates that the presence of a terminal oxidase is needed for full Pho induction.

Activation of Pho induction by *bd* expression does not require AbrB but does require reduced P_i levels and PhoR. To test whether *bd* expression in the *resD ydiH* mutant acts on Pho induction through the ResD-independent AbrB-dependent parallel pathway, we examined the effect of an *abrB* mutation on Pho induction in the Δ*resDE ydiH* background. A Δ*resDE ydiH abrB* triple mutant (MH7131) (Fig. 5A) retained the growth and Pho induction phenotypes of a Δ*resDE ydiH* double mutant (MH5887) (Fig. 5A), providing evidence that the AbrB path-

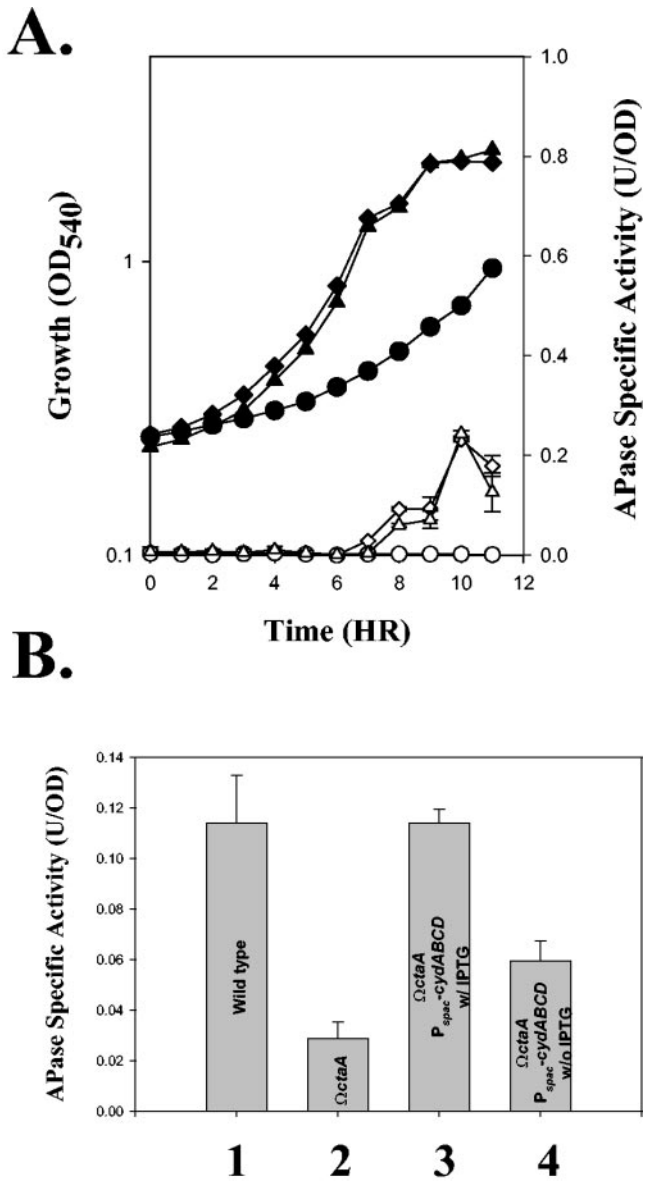

FIG. 4. Constitutive expression of *cydABCD* restores full Pho induction in *resDE* or *ctaA* strains. (A) Induction of cytochrome *bd* in a Δ*resDE* background is sufficient to restore Pho induction. Solid symbols, growth; open symbols, APase; diamonds, MH5857 (Δ*resDE ydiH*) with IPTG; triangles, MH5885 (Δ*resDE* P_{spac}-*cydABCD*) with IPTG; circles, MH5885 (Δ*resDE* P_{spac}-*cydABCD*) without IPTG. (B) Induction of cytochrome *bd* in a *ctaA* mutant background is sufficient to restore Pho induction. The results from each strain are the average APase specific activity from 20 cultures. Lane 1, JH642 (wild type) without IPTG; lane 2, MH7124 (Ω*ctaA*) without IPTG; lane 3, MH7128 (Ω*ctaA* P_{spac}-*cydABCD*) with IPTG; lane 4, MH7128 (Ω*ctaA* P_{spac}-*cydABCD*) without IPTG.

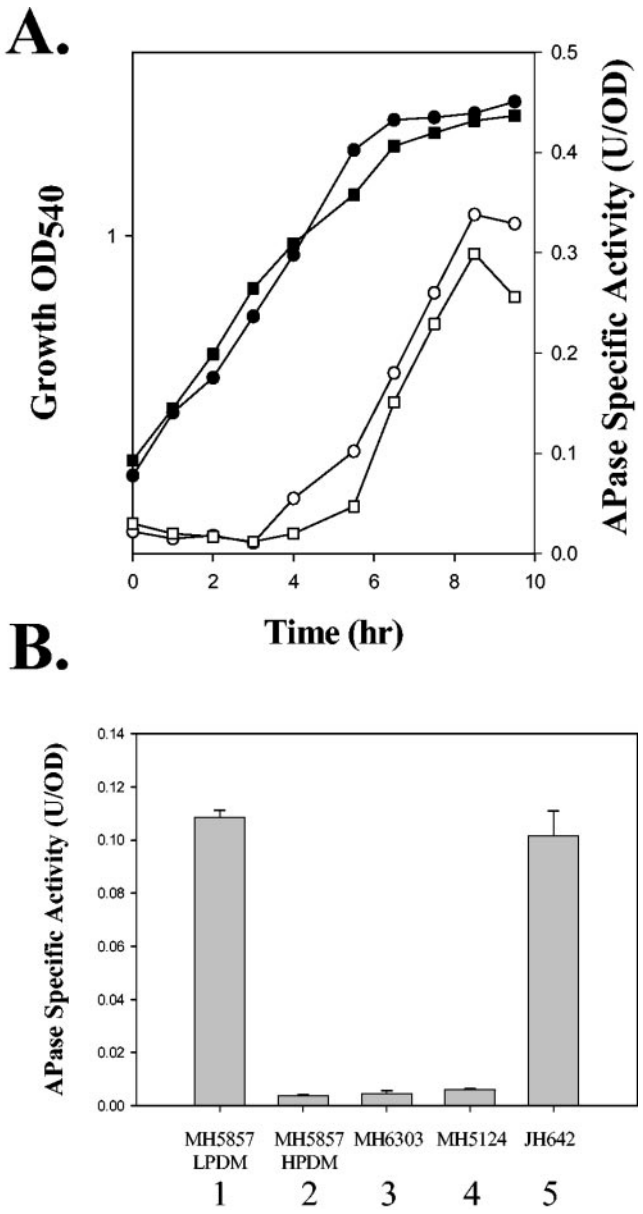

FIG. 5. Restoration of full Pho induction in a *resDE ydiH* strain does not require AbrB but does depend on reduced P_i levels and PhoR. (A) Pho induction in a *resDE ydiH* strain does not require AbrB. Solid symbols, growth; open symbols, APase specific activity; circles, MH5888 (*resDE ydiH*); squares, MH7131 (*resDE ydiH abrB*). (B) Pho induction in a *resDE ydiH* strain depends on reduced P_i levels and PhoR. The APase specific activities shown are the averages of duplicate readings from two cultures. Lane 1, MH5857 (*resDE ydiH*) grown in LPDM; lane 2, MH5857 (*resDE ydiH*) grown in HPDM; lane 3, MH6303 (*phoR* P_{spac}*-cydABCD*) grown in LPDM with IPTG; lane 4, MH5124 (Δ*phoR*) grown in LPDM with IPTG; lane 5, JH642 grown in LPDM.

way is not involved in suppression of the Δ*resD* phenotype on Pho induction in Δ*resDE ydiH* strains.

To confirm that Pho induction in a *resD ydiH* strain was phosphate starvation dependent, APase expression was assayed in strain MH5857 (*resD ydiH*) grown in LPDM and HPDM. MH5857 APase specific activity (Fig. 5B, lane 1) was comparable to that of the WT control (JH642) (lane 5) when grown in LPDM but failed to induce APase in the high-phosphate medium (HPDM) (Fig. 5B, lane 2), as did the *phoR*-negative control (MH5124) (lane 4) grown in LPDM, indicating Pho induction in a *resD ydiH* strain was phosphate starvation dependent.

To rule out the possibility that *bd* expression-dependent Pho induction was PhoR independent, we constructed a *phoR* P_{spac}*-cydABCD* strain, MH6303. APase was not induced in MH6303 (Fig. 5B, lane 3) grown in LPDM containing IPTG, indicating that expression of cytochrome *bd* from an IPTG-inducible promoter did not restore the Pho response in the absence of PhoR via activation of another kinase capable of PhoP phosphorylation.

The redox state of quinones affects PhoR autophosphorylation. The function of terminal oxidases in the cell is to oxidize the reduced menaquinone pool. The redox state of quinones has been reported to affect autophosphorylation of the histidine kinase of three signaling systems: ArcB (*E. coli*) (13), BvgS (*Bordetella pertussis*), and EvgS (*E. coli*) (5). The major form of quinones in *B. subtilis* is the menaquinone MK-7 (8). When the soluble analog, MK_3, was incubated with or without dithionite in the presence of [γ-^{32}P]ATP and purified *PhoR$^{231–589}$, a soluble NH_2-terminal truncation of PhoR (44), autophosphorylation of *PhoR was inhibited in the presence of dithionite-reduced MK_3 (Fig. 6) but not in the presence of MK_3 or dithionite alone compared to PhoR autophosphorylation in the absence of quinones. These in vitro data suggest that reduced quinones inhibit PhoR autophosphorylation. The finding that the addition of the membrane-penetrating reductant dithiothreitol (DTT) to low-P_i agar medium resulted in colonies with decreased expression of reporters of Pho induction (*phoA-lacZ*, *phoD-lacZ*, or *phoP-lacZ* fusions) compared to the same strains on the same medium without DTT is consistent with our in vitro data (data not shown).

DISCUSSION

In this report, we have shown that the expression of a terminal oxidase (cytochrome *bd*) in a Δ*resDE* background can bypass the requirement for ResD for full Pho induction. Previous reports (49) have demonstrated that ResD is required for 80% of the wild-type *B. subtilis* Pho response. To understand this mechanism, we studied Δ*resDE* strains bearing secondary mutations in *ydiH*. These strains exhibited wild-type levels of Pho induction despite the presence of a *resD* mutation (41). *ydiH* encodes a DNA-binding protein that acts as a negative regulator for the production of cytochrome *bd* (41). Our group had previously shown that a *resD* mutant did not produce terminal oxidases aa_3 and caa_3 (50) because ResD directly activates *ctaA* expression (35), which is required for conversion of heme O to heme A (51, 52). The absence of *cydA* expression in a *resDE* mutant compared to low-level expression during growth of the parental strain in LPDM (JH642) (Fig. 1) and the dependency of *cydA* expression on ResD (unpublished data) in the same strain grown in NSMPG (nutrient sporulation medium with phosphate buffer and fucose) indicate that a *resDE* strain is also deficient in cytochrome *bd* synthesis. Importance of terminal oxidases in Pho induction was supported by the observation that a *ctaA* strain also has reduced Pho induction and that expression of cytochrome *bd* in either a

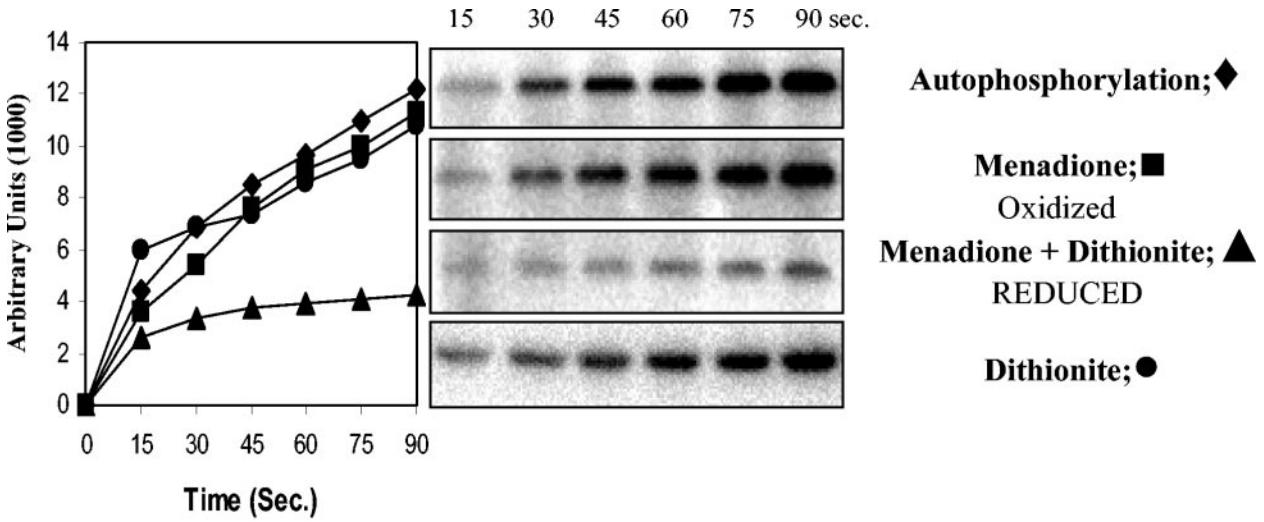

FIG. 6. Effect of MK_3 on the rate of autophosphorylation of the *$PhoR^{231–589}$ histidine kinase. The purified protein *$PhoR^{231–589}$ was incubated with [γ-^{32}P]ATP in the presence or absence of MK_3 and/or dithionite. The right panel shows the autoradiograms of the different SDS-polyacrylamide gel electrophoresis gels. In the left panel, quantification of the radioactive PhoR~P with time was measured with ImageQuant, and the results are expressed in arbitrary units.

Δ*resDE* background or a *ctaA* mutant background restored Pho induction to wild-type levels (Fig. 5).

The observation that the *ctaA* strain had reduced Pho induction is also important because it separated the Pho regulon dependency on terminal oxidases from the poor growth in LPDM. While there may be a number of reasons for the improved growth of a *ctaA* mutant strain compared to that of *resDE*, the most simple may be that all Res regulon genes except *ctaA* remain functional in a *ctaA* strain.

Among the Res regulon genes, *cydA* expression is likely important. Figure 2 showed that low-level expression of cytochrome *bd* takes place in the wild-type background but not in the Δ*resDE* background. Assuming the level of cytochrome *bd* production in the *ctaA* background is similar to that in the wild-type background, this difference may contribute to the difference in the growth phenotypes between the *resD* and *ctaA* strains. A role for ResD in control of other possible terminal oxidases remains untested, such as that with the *ythA ythB* genes (53).

Data presented here lead to the question of why ResD and the terminal oxidases are required for 80% of the wild-type Pho response. One possible role for ResD is in activation of transcription of the *phoPR* operon. However, previous work (Y. Chen and F. M. Hulett, unpublished data) showed that ResD does not footprint on the *phoPR* promoter at protein concentrations 20-fold higher than needed (56) for ResD binding on the *ctaA* promoter, leading us to believe that the role of ResD is not in transcriptional activation of *phoPR*. Analysis of an *abrB resDE ydiH* triple mutant that maintained the restored *resDE ydiH* Pho phenotype (Fig. 5A) ruled out the possibility that the bypass of the ResD role in Pho induction by terminal oxidase expression was via the upstream parallel AbrB pathway. Because constitutive *bd* expression does not induce the Pho response in a *phoR* mutant or in elevated P_i medium, the terminal oxidase ResD bypass for Pho induction retained dependency on PhoR and the requirement for a low-phosphate signal. The latter result indicates that the role of the terminal oxidases does not control the on-off signal received by PhoR. Rather, our data suggest that the role is likely modulation of the signal. Firstly, a Δ*resDE* strain showed 20% of the wild-type Pho induction upon phosphate depletion, indicating PhoR received the P_i deficiency signal but was incapable of full Pho induction. Secondly, constitutive expression of *cydABCD* encoding *bd* oxidase failed to induce Pho regulon genes during phosphate-sufficient growth, but upon P_i depletion supported full Pho induction similar to a *resDE YdiH* strain (Fig. 4A).

Postexponential expression of the *cydA* operon in the *resDE ydiH* mutant or wild-type strain has been observed in all media examined in which expression occurs (LB containing 0.5% glucose [41] and NSMPG [54; Schau et al., unpublished]) and LPDM (Fig. 1B), suggesting that there is regulation on the *cydA* promoter in addition to the YdiH repression, since the *cydA* promoter identified in a wild-type strain grown in NSMPG (54) was a putative σ^A promoter. A candidate regulator for the late induction of a σ^A promoter is a transition state regulator (48) that holds *cydA* silent until the transition from exponential to stationary growth, as has been shown or suggested for the *bc* complex (55) and caa_3 (53). Because LPDM was designed so that cells would enter stationary growth due to phosphate deficiency and *cydA* was expressed postexponentially in medium containing high levels of phosphate, it is reasonable that *cydA* would also induce postexponentially in LPDM, not in response to phosphate starvation but rather the same postexponential regulation exhibited in other media. Further work is required to determine the regulator(s) responsible for *cydA* postexponential expression.

That terminal oxidases are important for the level of Pho induction in *B. subtilis* provides an explanation for previous work which showed that a strain bearing a mutation in *nhaC*, which encodes a Na^+/H^+ antiporter, hyperinduces Pho regulon genes during phosphate deprivation (36). Other reports (24, 39, 42) had shown that K^+/H^+ and Na^+/H^+ antiporters, as well as protonophores, inhibit respiration in *B. subtilis*. If the Na^+/H^+ antiporter inhibits respiration, which results in reduc-

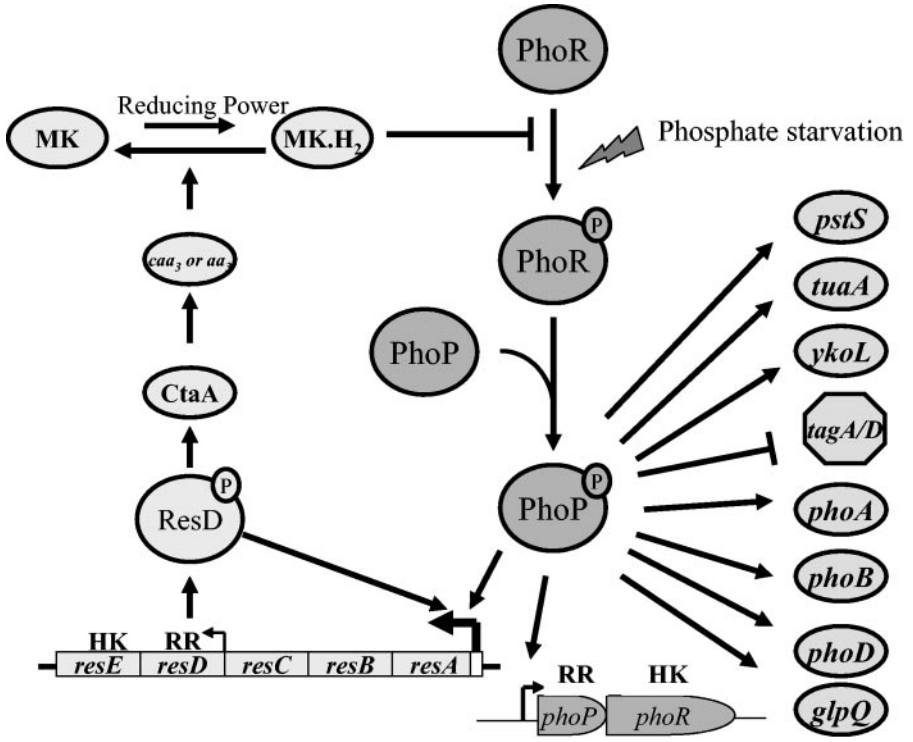

FIG. 7. Model depicting the present understanding of the feedback amplification loop between the ResD-ResE two-component signal transduction system and the PhoP-PhoR two-component system. Solid lines indicate that direct interaction has been demonstrated. Positive regulation is labeled with an arrow, while repression is labeled with a ⊥.

tion of terminal oxidase activity, which in turn inhibits Pho induction, then deletion of a gene responsible for the production of an Na^+/H^+ antiporter should improve respiration, leading to increased activity of the terminal oxidases causing hyperinduction of Pho induction, as was reported in the *nhaC* deletion strain.

Our data suggest that reduced quinones inhibit PhoR autophosphorylation. The mechanism of inhibition is currently under examination. Possible mechanisms include conformational changes in PhoR upon quinone binding or reduction of four potentially redox-sensitive cysteine residues in PhoR. Oxidized quinones have been shown to inhibit autophosphorylation in three systems that are involved in anaerobic energy production: ArcB (*E. coli*) (13), BvgS (*B. pertussis*), and EvgS (*E. coli*) (5). In contrast, PhoR is inhibited by reduced quinones and is involved in a positive feedback loop with the two-component regulators of aerobic respiration, ResD and ResE, under growth-limiting phosphate conditions (49). In the ArcB system it was recently reported (29) that the kinase inhibition involves two cytosol-located redox-active cysteine residues that participate in intermolecular disulfide bond formation, with the oxidative power for the reaction dependent on the redox state of the quinone pool. Because accumulation of reduced quinones, either by deletion of the *E. coli* terminal oxidases (21) or addition of DTT to the growth medium, led to activation of ArcA~P-activated reporter genes during aerobic growth, the redox state of quinones is believed to control the ArcB signal. In contrast, our current understanding of the *B. subtilis* Pho system suggests quinones control modulation of the phosphate deficiency signal. Our working hypothesis envisions that aberrant expression of cytochrome *bd* in the Δ*resDE ydiH* strain restores terminal oxidase function to a level that allows PhoR to autophosphorylate at wild-type levels and that the level of Pho induction ultimately depends on the level of PhoR autophosphorylation (Fig. 6).

Figure 7 illustrates our present understanding of the signal transduction loop between the PhoP/R system and the ResD/E system. PhoR autophosphorylates in response to a phosphate deficiency signal when phosphate levels in the medium decrease below 0.1 mM P_i. PhoP is phosphorylated in a PhoR-dependent manner, and low-level induction of PhoP regulon genes results. The *phoPR* operon is autoinduced, and the low levels of PhoP~P plus ResD (from the weak internal *resDE* promoter) are sufficient to activate the stronger *resA* promoter, thereby increasing cellular concentrations of ResD and ResE. ResD~P activates transcription of *ctaA* and *ctaB*, which are required for heme A biosynthesis, and thus *a*-type terminal oxidases aa_3 and caa_3. The terminal oxidases oxidize the reduced quinones to relieve inhibition of PhoR autophosphorylation to promote full Pho induction, completing the positive feedback loop between the Pho and Res systems under phosphate-limiting conditions.

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