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Regulators of Aerobic and Anaerobic Respiration in *Bacillus subtilis*

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Two *Bacillus subtilis* genes, designated *resD* and *resE*, encode proteins that are similar to those of two-component signal transduction systems and play a regulatory role in respiration. The overlapping *resD-resE* genes are transcribed during vegetative growth from a very weak promoter directly upstream of *resD*. They are also part of a larger operon that includes three upstream genes, *resABC* (formerly *orfX14*, -15, and -16), the expression of which is strongly induced postexponentially. ResD is required for the expression of the following genes: *resA*, *ctaA* (required for heme A synthesis), and the *petCBD* operon (encoding subunits of the cytochrome *bf* complex). The *resABC* genes are essential genes which encode products with similarity to cytochrome *c* biogenesis proteins. *resD* null mutations are more deleterious to the cell than those of *resE*. *resD* mutant phenotypes, directly related to respiratory function, include streptomycin resistance, lack of production of aa_3 or caa_3 terminal oxidases, acid accumulation when grown with glucose as a carbon source, and loss of ability to grow anaerobically on a medium containing nitrate. A *resD* mutation also affected sporulation, carbon source utilization, and Pho regulon regulation. The data presented here support an activation role for ResD, and to a lesser extent ResE, in global regulation of aerobic and anaerobic respiration in *B. subtilis*.

Regulation of anaerobic and aerobic respiration in *Escherichia coli* is controlled by four global systems. Genes necessary for optimal energy generation when O_2 is used as the electron acceptor are repressed by the ArcA-ArcB system in response to anoxia (25, 50). Generation of the signal requires terminal cytochromes, which suggests that ArcB detects the accumulation of a terminal electron carrier in the reduced state (24). A second transcription factor, Fnr, plays a role in adaptation to anaerobic and aerobic conditions. It is structurally similar to the cyclic AMP receptor protein, activates a number of genes involved in anaerobic respiration, and with ArcA, takes part in the activation of ArcA transcription (7). Two additional signal transduction systems are involved in nitrate regulation of anaerobic respiratory gene expression. NarL controls nitrate induction of nitrate respiration genes and repression of genes encoding alternate anaerobic respiratory proteins. NarP controls nitrite induction of several operons. Two homologous sensor proteins, NarX and NarQ, mediate both nitrate and nitrite signaling (53).

The regulation of respiration in *Bacillus subtilis* is not understood at the molecular level. However, a number of *B. subtilis* genes encoding biosynthetic enzymes or subunits for components of the respiratory chain have recently been cloned and characterized. Two operons are involved in heme biosynthesis. The *hemAXCBL* cluster encodes enzymes for the early steps of heme synthesis, from glutamyl-tRNA to uroporphyrinogen III (14, 41), while the *hemEHY* cluster encodes those for the late steps, UroIII to protoheme IX or heme B (13). HemX seems to have a role affecting the concentration of HemA (glutamyl-tRNA reductase) in the cells (44). *B. subtilis* expresses two kinds of heme-A-containing terminal oxidases, aa_3 and caa_3 (28), which catalyze quinol and cytochrome *c* oxidation, respectively. The subunits of caa_3 are encoded by *ctaCDEF* (43). Expression of caa_3 is not detectable during rapid growth but is maximal either during slow growth on nonfermentable carbon sources or postexponentially. The structural genes for aa_3 are *qoxA*, *qoxB*, *qoxC*, and *qoxD*, which specify subunits II, I, III, and IV, respectively (42). In contrast to caa_3, aa_3 is synthesized maximally during rapid growth and considered to be the major component in energy conversion during vegetative growth (43). *ctaB* and *ctaA* are genes involved in heme biosynthesis. CtaB is required for synthesis of heme O from heme B (57). CtaA was initially considered to have a regulatory role in the biosynthesis of aa_3 (34). More recently, it has been reported to have an enzymatic function in the conversion of heme O to heme A (57).

B. subtilis can grow anaerobically, using nitrate as the final electron acceptor (3, 7, 25, 36, 37, 39). Two nitrate reductase genes have been isolated; one for nitrate assimilation during aerobic growth encoded by *nasBC* (11, 36) and one required for nitrate respiration during anaerobic growth encoded by *narGHJI* (36). The *B. subtilis fnr* gene has been isolated and shown to be essential for anaerobic nitrate reductase activity (37).

During systematic sequencing of the *B. subtilis* genome by the European *Bacillus* genome sequencing project, two genes, *orfX17* and *orfX18*, encoding proteins with similarity to two-component signal transduction regulators in general and to the regulators of the *Bacillus* Pho regulon in particular, were discovered (49). In this paper, we report the functional characterization of the operon which includes these genes and genes whose products share similarity to cytochrome *c* biogenesis proteins. The two-component regulatory pair, designated ResD and ResE, has a positive role in global regulation of both aerobic respiration and anaerobic respiration.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1. Plasmids for this study, some of which are illustrated in Fig. 1, were constructed as follows. DNA fragments from the *res* operon region were identified by the base pair numbering in the sequence submitted to GenBank with accession number L09228 (49). (i) pES17 contains a 201-bp fragment of the *resA* promoter region (bp 18099 to 18300), which was amplified by PCR and cloned into the *Bam*HI-*Eco*RI sites of pDH32. (ii) pES24 contains a chloramphenicol resistance gene without a terminator on a 955-bp *Hin*cII-*Sma*I fragment from pJM105B (40), inserted into the *Ngo*MI site in *resA* on pES19. pES19 contains the *resA* promoter region, the entire *resA* gene, and 1.1 kb of the *resB* gene (bp 18099 to 19920) cloned into the *Bam*HI-*Eco*RI sites of pUC19. (iii) pES26 contains the pJM105B chloramphenicol resistance gene inserted into the *Bal*I site within the coding region of *resB* in pES19. (iv) pES33 was constructed by subcloning the *Bam*HI-*Eco*RI fragment from pES19, which contains the *resA* promoter and coding region and part of *resB*, into the *Bam*HI-*Eco*RI sites of pDH32. (v) pES28 contains an insertion in the *resC* gene which was constructed by cloning the pJM105B Cm^r gene into the *Eco*RV site within the coding region of the *resC* gene on pES20. pES20 contains the *resBC* intragenic region, the entire *resC* gene, and the *resD* internal promoter (bp 20444 to 21755) cloned into the *Bam*HI site of pUC18. (vi) pES31, which contains a mutation in *resD*, was created by cloning the truncated Cm^r gene from pJM105B into the *Ngo*MI site of the *resD* gene in pES21. pES21 contains the *resD* promoter region, the entire *resD* gene, and 650 bp of the *resE* gene (bp 21545 to 23086) in the *Hin*cII site of pUC18. (vii) pES50 contains a 782-bp *Nae*I deletion within *resE* into which a Cm^r gene was inserted in plasmid pES46. pES46 contains the 3′ end of *resD* and the *resE* gene (bp 22321 to 24222) amplified by PCR and cloned into the *Pst*I site of pUC18. (viii) pES55 contains the P*spac* promoter in the same orientation as *resABC* in pDH88. To construct pES55, the *resABC* insert in pES54 was released by digestion with *Sph*I and subcloned into the *Sph*I site on pDH88. pES54 contains the *Spe*I-*Xba*I Klenow-treated fragment of pES48 in the *Hin*cII site of pUC19. pES48 contains *resABC* (bp 18229 to 21755) amplified by PCR and cloned into the pCRII vector. (ix) pGS10 contains a 761-bp internal fragment of *ctaA* (bp 199 to 960 [34]), amplified by PCR and cloned into the *Eco*RI site of pJM103. (x) A 397-bp DNA fragment internal to *resD* (bp 21812 to 22209) was generated by PCR and cloned into the *Sma*I site of pJM103 (10). (xi) pRC18 was constructed by ligating a PCR-generated 415-bp (bp 22771 to 23186) DNA fragment internal to *resE* into the *Sma*I site of pJM103. (xii) pRC22 contains a tetracycline resistance gene from pBC16 (26) flanked by 415 bp from *resE* (bp 22771 to 23186) and 514 bp of *resCD* (bp 21546 to 22060), such that transformation of the linearized plasmid into a recipient strain results in a deletion of the 3′ terminus of *resD* and the 5′ terminus of *resE* with the tetracycline resistance gene between the remaining portions of *resDE*. (xiii) pRC1234 contains a 210-bp fragment of the *resD* internal promoter region (bp 21545 to 21755), which was amplified by PCR and cloned into the *Bam*HI-*Eco*RI sites of pDH32.

Media, growth conditions, and enzyme assays. For the induction of sporulation, the cells were grown in modified Schaeffer's sporulation medium (SSG) (29). For phosphate starvation induction of alkaline phosphatase (APase) or the *resA* promoter fusion, the cells were cultured in the low-phosphate defined medium (LPDM) as described by Hulett et al. (18). APase specific activity was determined as described previously (18). β-Galactosidase specific activity was determined according to the method of Ferrari et al. (9). β-Galactosidase specific activity was expressed in units per milligram of protein). The unit used was equivalent to 0.33 nmol of ortho-nitrophenol produced per min. TBAB is 33% tryptose blood agar base (Difco) and is supplemented with 0.5% glucose (TBABG). Agar (1.5%) was added for plates. Spizizen's minimal salts medium plates (51) were used for carbon utilization studies.

Culture growth for phosphate or nitrogen depletion was done as follows. A 12-h culture of *B. subtilis* MH5201 grown in LPDM containing 1 mM K_2HPO_4 was used to inoculate a fresh culture of LPDM without K_2HPO_4 (for P_i starvation) or LPDM with 1 mM phosphate but without ammonium sulfate (for N starvation) to an A_{540} of 0.3. For both experiments, growth and β-galactosidase specific activity were measured hourly. When the β-galactosidase specific activity of either culture was ≥1200 U/mg $protein^{-1}$, the culture was divided into two flasks. The limiting nutrient, either phosphate (final concentration, 1 mM K_2HPO_4) or nitrogen [final concentration, 30 mM $(NH_4)_2SO_4$], was added to one of the two flasks, while the other flask remained starved. Growth and β-galactosidase specific activity of all flasks were monitored for another 3 h.

Anaerobic growth on plates was carried out in a BBL GasPak jar with BBL GasPak Anaerobic System chemicals according to the manufacturer's instructions. Anaerobic growth in liquid culture was carried out by the method of Glaser et al. (11) in 2XYT broth supplemented with 0.5% glucose, with and without 0.2% KNO_3. Nitrate reductase was assayed by the method of Glaser et al. (11). Nitrate reductase specific activity was defined as nanomoles of nitrite produced per milligram of protein per minute. The amount of nitrite was determined by a standard curve by using sodium nitrite.

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 early-stationary-phase cultures grown in Luria broth (LB). Deoxyribonuclease and ribonuclease were omitted from the lysis procedure. Solubilization of cytochromes and analysis of difference absorption spectra were done as described by Mueller and Taber (34). Difference absorption spectra (dithionite reduced minus oxidized) of laurylmaltoside cytoplasmic extracts were recorded at room temperature at a scan speed of 5 nm/s with a Hitachi U-2000 spectrophotometer. The membrane extracts were adjusted to 1.0 mg of protein per ml with 50 mM Tris-HCl–1.0 mM EDTA (pH 7.4), and 1.0-ml volumes were placed in sample and reference cuvettes. Reduction and oxidation were accomplished as previously described (34). Protein content was determined according to instructions supplied by Bio-Rad.

Identification of transcription initiation sites. *B. subtilis* MH5201 (*resA-lacZ*) was grown in LPDM or SSG to induce expression under the different conditions. Induction of the *resA* promoter was monitored by measuring β-galactosidase activity. *B. subtilis* MH5201 was cultured in LPDM, SSG, and LB to obtain cells for RNA extraction used in the analysis of the *resDE* promoter. Total RNA was extracted from the cells by the method of Ebbole and Zalkin (8), with modifications according to the method of Chesnut et al. (6). Transcription initiation sites were mapped by primer extension (23), using two different oligonucleotide primers for each promoter. For *resA*, the first primer, FMH165, was complementary to nucleotides 18300 to 18282, and the second primer, FMH176, was complementary to nucleotides 18340 to 18324 of the *resA* sequence. For primer extensions of the internal promoter 5′ of *resD*, the first primer, FMH121, was complementary to nucleotides 21766 to 21734 of the *resD* sequence. End-labelled primers (12 ng) were annealed to RNA (10 μg from phosphate-starved cells or 30 μg from sporulating cells) in 250 mM KCl–40 mM Tris-HCl (pH 8.3) at 42°C and 5 mM dithiothreitol–0.3 U of RNasin (Promega) μl^{-1} for 16 h at 37°C. Primers were extended with avian myeloblastosis virus reverse transcriptase (Promega) according to the manufacturer's specifications, and extended products were separated by electrophoresis in 6% polyacrylamide–8 M urea gels. The RNA preparations and Northern (RNA) hybridization experiments for the cytochrome *bf* complex genes were done as described earlier (1).

Spore resistance. The percentage of heat-, lysozyme-, or chloroform-resistant spores was determined by the method of Nicholson and Setlow (38).

Electron microscopy. Cells (1 ml) from a 36-h growth in SSG were washed three times with 0.1 M cacodylate buffer (pH 7.2; buffer A) and suspended in a 2.5% glutaraldehyde solution in buffer A. The cells were fixed at room temperature for 2 h, collected by centrifugation, and washed three times with buffer A. The cells were osmium treated in a 1% osmium tetroxide solution in buffer A at 4°C for 2 h; this was followed by three buffer A washes at 4°C. The cells were brought to room temperature and dehydrated by suspension for two 10-min periods in each of a graded series of ethanol (25, 50, 75, and 100%). The cells were infiltrated with propylene oxide (two changes, 10 min each) and suspended in 1 part propylene oxide to 1 part Spurr's resin before overnight mixing by rotation. Infiltration on the rotator was continued with three changes of pure resin over 24 h. Polymerized blocks were trimmed and sectioned with a Reichert-Jung Ultracut E ultramicrotome on a DDK diamond knife. Silver–silver-gold sections were collected on 200 hex mesh copper grids. Sections were made electron dense with uranyl acetate and lead citrate and photographed with a JEOL 1200 EX transmission electron microscope.

RESULTS

Genes and products of the *res* operon. A map of the region encoding *orfX14* to *orfX18* (49), designated *resA* to *resE* hereafter, is shown in Fig. 1.

ResA shows significant similarity to cytochrome *c* biogenesis proteins (2, 3) and contains a block of amino acids identified as the thioredoxin family active site (15). ResB is a very hydrophobic protein with no homology with proteins in databases. ResC contains the motif W-G-X-X-W-X-W-D, which has been purported to play a role in heme binding (58). ResD and ResE show significant similarity to a family of two-component signal transduction proteins, the greatest similarity being to the Pho regulators of *B. subtilis* (20, 45, 46).

***resA*, *resB*, and *resC* are essential genes.** In an effort to determine the functions of ResA, ResB, and ResC, we used a chloramphenicol cassette designed to produce nonpolar mutations (40) and used successfully in the analysis of a 26-kb chemotaxis operon (27). Null mutations in *resA*, *resB*, or *resC* could not be constructed. Numerous attempts to interrupt *resA* with pES24 or *resB* with pES26, by transformation of the *B. subtilis* strains JH642 or 168 with linearized plasmid DNA, did not yield any transformants. Such recombinants should have a terminatorless chloramphenicol resistance gene within the *resA* or *resB* gene, allowing transcription of downstream genes in the operon (40). Attempts to integrate circular plasmids by a single crossover event were also unsuccessful. However,

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or antibiotic resistance (kb)	Source or reference
B. subtilis strains		
168	*trpC2*	Laboratory strain
IA473	*trpC2 bfmB1 strC2*	BGSC[a]
JH642	*pheA1 trpC2*	J. A. Hoch
MH1000	*pheA1 trpC2 amyE*::pDH32 Cm^r	Laboratory strain
MH5004	*pheA1 trpC2 amyE*::pRC1234 Cm^r	This study
MH5061	*pheA1 trpC2 resE*ΩpRC18 Cm^r	This study
MH5081	*pheA1 trpC2 resDE*::pRC22 Tet^r	This study
MH5201	*pheA1 trpC2 amyE*::pES17 Cm^r	This study
MH5202	*pheA1 trpC2 amyE*::pES17 Cm^r *resDE*::pRC22 Tet^r	This study
MH5229	*pheA1 trpC2 amyE*::pES17 Sp^r	This study
MH5257	*pheA1 trpC2 ctaA*ΩpAI600 (*ctaA*$^+$) Cm^r	This study
MH5260	*pheA1 trpC2 resD*::pES31 Cm^r	This study
MH5262	*pheA1 trpC2 ctaA*ΩpAI600 (*ctaA*$^+$) Cm^r *resDE*::pRC22 Tet^r	This study
MH5263	*pheA1 trpC2 amyE*::pES17 Cm^r *resD*::pES31 Sp^r	This study
MH5266	*pheA1 trpC2 resC*ΩpES28 Cm^r	This study
MH5283	*pheA1 trpC2 ctaA*ΩpAI600 (*ctaA*$^+$) Cm^r *resD*::pES31 Sp^r	This study
MH5293	*pheA1 trpC2 amyE*::*resAB* Sp^r	This study
MH5418	*pheA1 trpC2 resE*::pES50 Cm^r	This study
MH5423	*pheA1 trpC2 amyE*::pES17 Sp^r *resE*::pES50 Cm^r	This study
MH5450	*pheA1 trpC2* P*spac-resABC*ΩpES55 Cm^r	This study
MH5451	*pheA1 trpC2* P*spac-resABC*ΩpES55 Cm^r *resDE*::pRC22 Tet^r	This study
MH5454	*pheA1 trpC2 amyE*::pES17 Sp^r P*spac-resABC*ΩpES55 Cm^r	This study
MH5455	*pheA1 trpC2* P*spac-resABC*ΩpES55 Sp^r	This study
MH5456	*pheA1 trpC2 ctaA*ΩpAI600 (*ctaA*$^+$) Cm^r *resE*::pES50 Sp^r	This study
MH5458	*pheA1 trpC2 amyE*::pES17 Sp^r P*spac-resABC*ΩpES55 Cm^r *resDE*::pRC22 Tet^r	This study
MH5459	*pheA1 trpC2 ctaA*ΩpAI600 (*ctaA*$^+$) Cm^r P*spac-resABC*ΩpES55 Sp^r	This study
MH5465	*pheA1 trpC2 ctaA*ΩpAI600 (*ctaA*$^+$) Cm^r P*spac-resABC*ΩpES55 Sp^r *resDE*::pRC22 Tet^r	This study
MH5466	*pheA1 trpC2 ctaA*ΩpGS10 (*ctaA*) Cm^r	This study
RB972	*trpC2 ctaA*ΩpAI600 (*ctaA*$^+$) Cm^r	H. Taber
E. coli strains		
SURE	*mcrA* Δ*mcrBC-hsdRMS-mrr)171 endA1 supE44 thi-1* λ^- *gyrA96 relA1 lac recB recJ sbcC umuC*::Tn*5*(Kan^r) *uvrC* [F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10*(Tet^r)]	Stratagene
DH5α	*F′ ϕ80 dlacZ* ΔM15 Δ(*lacZYA-argF*) *U169 recA1 endA1 hsdR17* (r^- m^+) *supE44 med*λ^- *thi-1 gyrA relA1*	Laboratory strain
Plasmids		
pCRII	Amp^r Kan^r (3.9)	Invitrogen
pDH32	Amp^r Cm^r (9.9)	47
pDH88	Amp^r Cm^r (6.1)	D. J. Henner
pES17	Amp^r Cm^r (10.1)	This study
pES19	Amp^r (4.5)	This study
pES20	Amp^r (4.1)	This study
pES21	Amp^r (4.2)	This study
pES24	Amp^r Cm^r (5.4)	This study
pES26	Amp^r Cm^r (5.4)	This study
pES28	Amp^r Cm^r (5.0)	This study
pES31	Amp^r Cm^r (5.2)	This study
pES33	Amp^r Cm^r (11.7)	This study
pES46	Amp^r (4.6)	This study
pES48	Amp^r Kan^r (7.5)	This study
pES50	Amp^r Cm^r (6.1)	This study
pES54	Amp^r (6.4)	This study
pES55	Amp^r Cm^r (9.8)	This study
pGS10	Amp^r Cm^r (4.5)	This study
pJL62	Amp^r Sp^r Tet^r (6.55)	I. Smith
PJM103	Amp^r Cm^r (3.7)	J. A. Hoch
pMI1101	Amp^r Cm^r (9.0)	T. Msadek
pRC17	Amp^r Cm^r (4.1)	This study
pRC18	Amp^r Cm^r (4.1)	This study
pRC22	Amp^r Tet^r (6.2)	This study
pRC1234	Amp^r Cm^r (10.1)	This study

[a] *Bacillus* Genetic Stock Center.

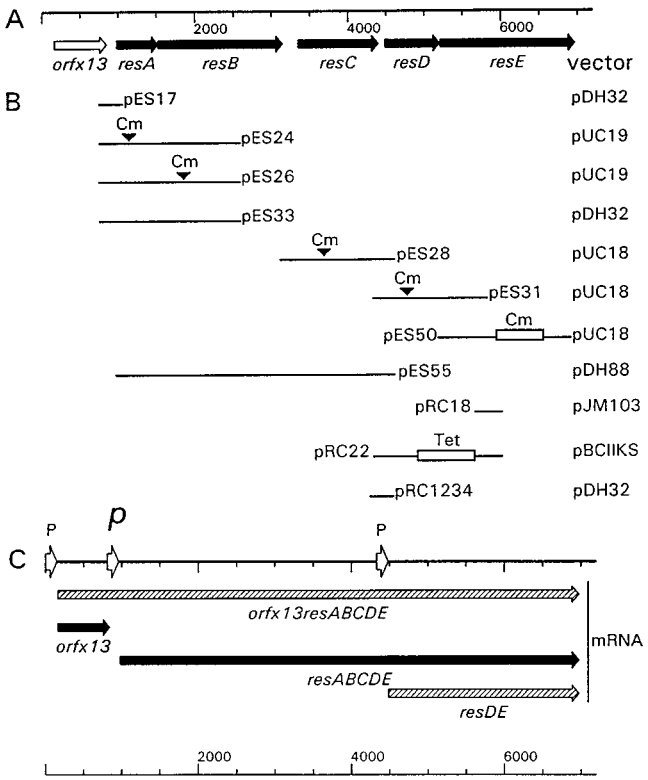


FIG. 1. Map of the *res* region in the *B. subtilis* chromosome and in different plasmids, including transcriptional units in the region. (A) The wild-type *resABCDE* gene cluster at 210° on the *B. subtilis* map. The scale is given in base pairs. The solid arrows identify the sizes and layout of the genes in the *res* operon. The open arrow positions the upstream gene, *orfX13*. (B) DNA inserts in plasmids used in characterization of the *res* operon. For details of the plasmid constructions, see Materials and Methods. The thin solid line represents DNA cloned within the plasmid named directly after the line. The vector used in each construction is indicated at the extreme right. ▼, position of insertion of a terminatorless Cm^r gene from pJM105B into the DNA clone. □, a deletion in the *resDE* genes and the insertion of the Tet^r gene (pRC22) or a deletion in the *resE* gene and the insertion of a Cm^r gene (pES50). (C) Promoter positions and lengths of transcriptional units in the *res* chromosomal region. The open arrows identify the promoters as follows: P, the major *res* promoter; p, the weaker internal promoter; and the promoter for *orfX13*, implied from previous Northern analysis (1), upstream of the *res* genes. Abundant transcripts are indicated by thick solid arrows. Minor transcripts are represented by hatched arrows.

transformants with either plasmid were obtained readily when *resA* and *resB*, together with their upstream promoter, were first integrated at the *amyE* locus of JH642, to create a merodiploid strain (MH5293). Null mutations of *resC*, resulting from integration of linearized pES28, also appeared to be lethal. However, transformants could be obtained from a single-crossover integration (Campbell integration) of pES28 which results in one intact copy and one mutated copy of *resC*. Interestingly, these transformants were obtained only when the crossover event positioned the uninterrupted copy of *resC* adjacent to *resB* (data not shown). In this case, the *resA* promoter could direct transcription of *resABC* but not *resDE*. Thus, it appears that three genes in this operon, *resA*, *resB*, and *resC*, are essential for cell survival under the conditions tested.

Phenotypic characterization of *resD*, *resE*, and *resDE* mutants. Three *B. subtilis* strains, MH5260 (*resD*), MH5418 (*resE*), and MH5081 (*resD resE*), were used to determine the effects of mutations in *resD* and *resE*. The strains were constructed by double crossover of linearized pES31, pES50, or pRC22 (Fig. 1) into the JH642 chromosome. They display pleiotropic phenotypes concerning the following traits: streptomycin resistance, cytochrome spectrum, anaerobic respiration, colony morphology, carbon source utilization, sporulation, lysozyme sensitivity, and APase productivity.

Strains containing a *resD* and/or a *resE* mutation grew poorly on complex peptone medium (TBAB) and, with time, developed many opaque papillae which contained cells that retained the original mutations but had acquired compensatory mutations. The presence of 6-carbon sugars (glucose or fructose) in TBAB medium restored normal colony growth to *resD* mutant strains (MH5081 and MH5260), but colonies were distinctly pink. Use of lactate, pyruvate, or succinate as a carbon source failed to restore normal growth. Neither *resD* nor *resDE* mutants grew on Spizizen's minimal medium plates supplemented with succinate, lactate, or pyruvate as the sole carbon and energy source, but they did grow, albeit less well than the JH642 parental strain, when glucose was added. The colony phenotype of the *resE* mutant, in all cases, was slightly less severe than that of the *resD* or *resDE* mutant strains.

On the basis of Northern blot analysis (1), the *resDE* genes were included in an operon with genes which may function in respiration. The growth phenotypes of a *resDE* mutant described above were similar to those reported by Mueller and Taber for *ctaA* mutants which are deficient in cytochrome aa_3

TABLE 2. Comparison of phenotypic properties of *ctaA* and *res* mutants

Strain and relevant genotype	Red pig[a]	Acid ac[b]	Str^r (mm)[c]	TMPD[d]	*a*-type cytochrome[e]	Spo (%)[f]	Anaerobic growth[g]
JH642	−	−	2.5	++	+	100	++
MH5466 (*ctaA*)	+	+	0.2	−	−	<0.001	++
MH5081 (*resDE*)	+	+	0.1	−	−	0.01	−
MH5418 (*resE*)	±[h]	−	2.5	+	+	50	±
MH5266 (*resC*Ω)	+	±	0.3	−	ND[i]	0.01	+
MH5451 (P*spac resABC resDE* − IPTG)	+	+	0.5	−	ND	ND	−
MH5451 (P*spac resABC resDE* + IPTG)	+	+	1.5	−	ND	30	−
IA473 (*strC2*)[j]	−	−	1.0	+[k]	+[k]	ND	+

[a] Red pig, pink colony on TBABG medium.
[b] Acid ac, accumulation of acid in glucose-containing medium.
[c] Streptomycin resistance as determined by halo size around a 10-μg streptomycin disk.
[d] Ability to oxidize artificial electron donor TMPD.
[e] *a*-type cytochrome c, cytochrome aa_3, and caa_3 deficient.
[f] Percentage of heat-resistant spores compared with wild-type strain (JH642).
[g] Anaerobic growth in medium containing nitrate.
[h] resE mutant colonies develop pink centers on TBABG medium.
[i] ND, not determined.
[j] strC, an uncharacterized conditional mutation which causes reduced expression of *ctaA* (34, 50).
[k] Reported in reference 50.

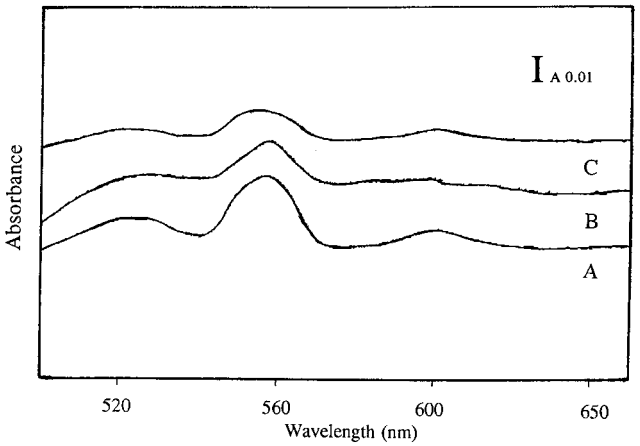


FIG. 2. Room-temperature (reduced minus oxidized) difference spectra of solubilized *B. subtilis* membranes. (A) Parent strain, JH642. (B) *resD* mutant, MH5260. (C) *resE* mutant, MH5418. Cells were grown in LB. The protein concentration in each membrane sample was 1 mg/ml.

synthesis (34). We asked if other phenotypes of the *ctaA* mutant were shared by the *resDE* mutants (Table 2). *resD* and *resDE* strains accumulated acid when grown in medium with glucose and were more resistant to streptomycin than was the parent strain (Table 2). Streptomycin-resistant mutants that are incapable of cytochrome aa_3 synthesis can be distinguished from conditional streptomycin mutants such as *strC* (34, 52) on the basis of their inability to oxidize the artificial electron donor, TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine). Mutants which produced cytochrome aa_3 levels lower than 30% of normal cellular levels failed to oxidize TMPD (34). *resD* and *resDE* mutant strains (Table 2) failed to oxidize TMPD. *resE* mutants oxidized TMPD less well than did the parent strain.

Analysis of difference light absorption spectra of solubilized membranes from the parent strain (JH642) and the *res* mutant strains revealed that the *resD* and *resDE* strains completely lacked both cytochrome aa_3-600 and caa_3-605 (Fig. 2) but accumulated elevated levels of cytochrome *b*. The cytochrome spectra of the *resE* mutant membrane showed that low concentrations of the *a*-type cytochrome terminal oxidase(s) were produced with no accumulation of cytochrome *b*.

Light microscopy of the *resD* and *resDE* mutant strains (MH5260 and MH5081, respectively) cultured in SSG showed that in approximately 50% of the cells, sporulation was initiated and progressed to the phase-bright-forespore stage with no release of free spores. Although about 50% of the *resD* mutant cells produced forespores, a very low percentage of cells produced spores resistant to heat (<0.001%), chloroform (<0.5%), or lysozyme (<0.5%), while 40 to 60% of the parental cells produced spores resistant to these treatments. Transmission electron microscopy of *resDE* double-mutant cells (MH5081) (Fig. 3) showed that most of the sporulating cells had reached stage IV to V but did not develop further, lacking a spore coat, though the cortex was normal.

Vegetative cells of *resD*, *resE*, and *resDE* mutant strains, cultured in SSG or in LPDM, were much more sensitive to lysozyme than the parent strain (JH642). Treatment with 250 μg of lysozyme per ml of cell culture resulted in complete lysis of the *res* mutant strains within 10 min, while there was no change in the optical density of parental cells after identical treatment.

The data described above suggest that the *resDE* genes have a role in aerobic respiration. We asked if they also had a role in anaerobic growth. JH642 can grow anaerobically on TBAB, TBABG, or Spizizen's minimal medium plates supplemented with 0.2% nitrate. *resDE* and *resD* mutants could not grow anaerobically on medium supplemented with nitrate; *resE* mutants showed slight growth.

Because of the similarity between the ResDE and PhoPR proteins, we asked whether the Res proteins regulate Pho regulon genes. Mutations in *resE* reduced phosphate starvation-induced APase specific activity to from 30 to 40% and mutations in *resD* reduced phosphate starvation-induced APase specific activity to 10% of wild-type levels.

Genes dependent on ResDE. (i) *resABCDE* operon. A transcript, approximately 7 kb in length, was detected in Northern blots of RNA extracted from wild-type cells with probes from

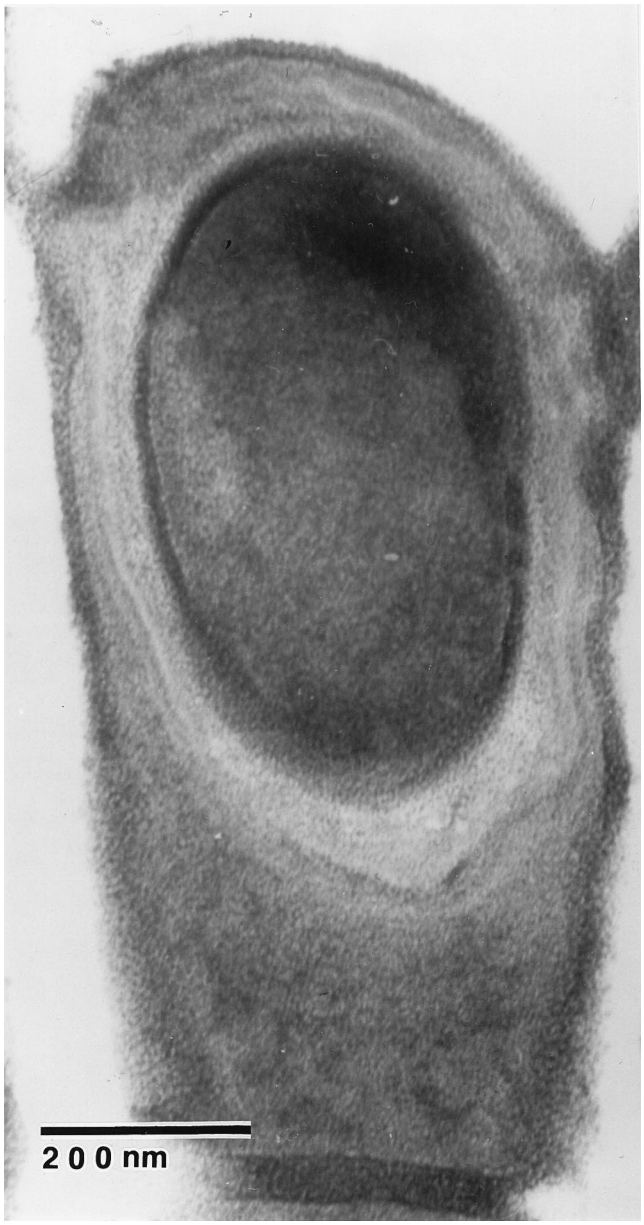
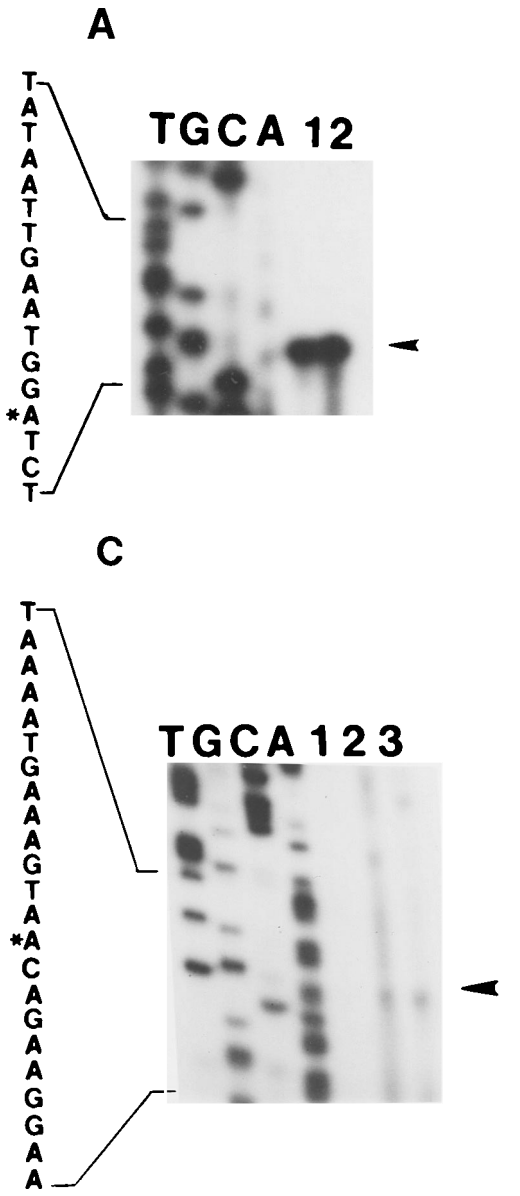


FIG. 3. Sporulation development blocked at stage V in *resDE* mutant. Over 50% of the *resDE* mutant cells (MH5081) produced unreleased forespores defective in coat formation.



B

ResA PROMOTER

CGATGCCAGAGAGTTACGCCTCACGAAGTAAAAAGGC
TCAGAGCACTGGCGGATCACGGTAAAAACGCTTTCTA
AATTTCACATAACCTTCAAAAAGTAAGAAATGTGAAA
-10 *
CGTGCAATGATATAATTGAATGGATCTGCAATAGGGG
MET
GAAGGGGGCAGTGACAATGAAGAAAAAAAGGCGTTTA
TTCATTCGGACC

D

resD promoter

GCCGTAATCGGTTTTGCTATTATTATGTTTAATTTAA
TATTCGTAAATCTTGTGTCTTGCCGGACTTCATTCTT
ATGCATAGTCCTTTGAAATCCTTTGAAAGCGACAGTT
-35 -10 *
CTCAAACTTTCTCACGATTTGATAAAAATGAAAGTAAC
MET
AGAAGGAAAGCAGGGGAAAACATGGACCAAACGAACG
AAACAAAAATATTAGTAGTTGATGACGAAGCCAG

FIG. 4. Primer extension analysis of *resA* and *resD* promoters. (A) Identification of transcriptional initiation site of *resA* promoter. The end-labeled primer was annealed to RNA from post-exponential-stage cultures grown in LPDM (lane 1) and SSG (lane 2). Lanes T, G, C, and A are sequencing ladders generated by annealing the same end-labeled primer to a plasmid containing the 5′ end of *resA* and extending it with Sequenase (United States Biochemical Corp.). The asterisk indicates the base to which the primer extension product maps. (B) Promoter region sequence of *resA*. MET, above GTG marks the putative translation start site of ResA. The transcription start site, A, is double underlined, in boldface print, and marked with an asterisk. The putative −10 region is underlined and in boldface print. The underlined sequences at the extreme 5′ and extreme 3′ ends identify the primers used to amplify the sequence for cloning. The 3′ primer was one of the two primers used in primer extension. TAA identifies the stop codon for the upstream gene. (C) Primer extention analysis of *resD* promoter. Same as for *resA* (panel A) except that RNA from cells cultured in LB was also used (lane 3). (D) Promoter region sequence of *resD*. Promoter elements are as identified for the *resA* promoter (panel B).

resA, *resB*, *resC*, *resD*, or *resE*. Primer extension was used to identify the *resA* promoter responsible for transcription of the entire *res* operon. RNA was isolated from post-exponential-stage cultures grown in LPDM and SSG. The start site of transcription was identical under both growth conditions, with either of the two primers complementary to sites within the coding region of *resA* (Fig. 4A). The consensus −10 sequence for a sigma A promoter, TATAAT, is located 10 bp upstream of the apparent transcriptional start site. No consensus sequence for the −35 region was found (Fig. 4B).

To study the expression of the *resA* promoter, it was cloned in front of the promoterless *lacZ* gene in pDH32, and the resulting plasmid (pES17) was linearized and integrated at the nonessential *amyE* locus of *B. subtilis* (JH642) cells. β-Galactosidase production from the promoter located 5′ of *resA* (MH5201) was induced as culture growth was limited because of phosphate or nitrogen depletion in LPDM (Fig. 5) and postexponentially as the culture entered stationary phase because of nutrient exhaustion in SSG (Fig. 6A).

To investigate the possibility that ResD and/or ResE might autoregulate the transcription of the *res* operon, pES17 was linearized and introduced by transformation into cells of the *resDE* mutant (MH5081). To construct the *resD* or *resE* mutant strains with *resA-lacZ* fusions, MH5201 was transformed with

pJL62 to interrupt the chloramphenicol resistance gene with a spectinomycin resistance gene (strain MH5229). MH5229 (*resA-lacZ*) was transformed with chromosomal DNA from MH5260, constructing MH5263 (*resA-lacZ resD*), and it was transformed with chromosomal DNA from MH5418, constructing MH5423 (*resA-lacZ resE*). The resulting strains, MH5202 (*resDE*), MH5263 (*resD*), MH5423 (*resE*), and MH5201 (the parent strain), were grown in SSG and assayed for β-galactosidase activity as a measure of transcription from the *resA* promoter. As shown in Fig. 6A, β-galactosidase activity was at low levels during exponential growth and strongly induced postexponentially in the parent strain but was barely detectable in the *resD resE* mutant. The β-galactosidase activity from a *resA* promoter in a *resD* mutant strain was identical to that in the *resDE* double mutant (data not shown). In the *resE* mutant strain, the expression of the *resA* promoter was off during exponential growth and was induced to about 5% of normal levels during postexponential growth. This suggests that expression of the *resA* operon is dependent on ResD for induction and is only slightly less dependent on ResE.

(ii) *resDE* suboperon. A second minor transcript of 2.4 kb hybridized to *resD* or *resE* probes (1). These data suggested that while *resD* and *resE* are part of a larger operon that includes genes encoding proteins homologous to those involved in respiration, the two genes also compose a transcriptional unit.

To identify the promoter responsible for the independent transcription of *resD* and *resE*, RNA was extracted from wild-type cells grown in LPDM, SSG, and LB. Primer extension was performed on all RNAs with a primer complementary to a site within the *resD* coding region. Prominent bands were observed 25 bases 5′ of the putative AUG initiation site on RNA from cells grown in SSG and LB (Fig. 4C). This would position a consensus sequence (5 of 6 bases) for a sigma A promoter at the −10 region, TAAAAT, and a poor consensus sequence (4 of 6 bases) at the −35 region, TTCTCA (Fig. 4D). Weaker bands were also present and were interpreted as the result of polymerase pausing on the longer transcript initiated at the *resA* promoter.

The promoter region upstream of *resD* was cloned in front of the *lacZ* gene in pDH32. The resulting plasmid (pRC1234) was linearized and integrated at the *amyE* locus of *B. subtilis*. β-Galactosidase production from the putative promoter upstream of *resD* (MH5004) was induced during epxonential growth in cells grown in SSG, but the specific activity decreased as the cell entered stationary phase (Fig. 7). The strength of this internal promoter is quite low compared with that of the *resA* promoter.

(iii) *ctaA*. Since the ResD-ResE two-component system seemed to be respiration related and many of the phenotypes of the *resDE* mutant were similar to those of a *ctaA* mutant (55), we asked if *ctaA* was controlled by ResD-ResE. CtaA was identified as a protein required for cytochrome aa_3 biogenesis (a terminal oxidase in the *B. subtilis* electron transport chain), for growth when lactate is the sole carbon and energy source and for sporulation (34). More recently it has been suggested that CtaA is a heme O monooxygenase, responsible for the synthesis of heme A from heme O (56). Chromosomal DNA from *B. subtilis* RB972 (35) was used to introduce the *ctaA-lacZ* promoter fusion into the parent strain (JH642), resulting in MH5257. For construction of MH5262 (*ctaA-lacZ resDE*), MH5257 was transformed with chromosomal DNA from MH5081, selecting for Tet^r. To construct MH5456 (*ctaA-lacZ resE*), MH5418 (*resE*Δ Cm) was transformed to Sp^r with pJL62; a resulting transformant was made competent and transformed with chromosomal DNA from MH5257 (*ctaA-lacZ*). The resulting strains were grown in SSG and assayed for β-galactosidase activity. As shown in Fig. 6B, expression in the parent strain (MH5257) was at a low level during exponential growth, increased significantly during the late exponential stages, and reached a peak of 350 U after 4 h into stationary phase. In the *resD resE* mutant MH5262, β-galactosidase activity remained near zero throughout the 12-h growth (Fig. 6B) as it did also in the *resD* mutant MH5283 (data not shown). Promoter function was only slightly less than that in the parent strain (MH5257) during exponential growth of the *resE* mutant MH5456, but it decreased to zero as *ctaA* expression was postexponentially induced in the parent strain. Although the *resA* promoter is stronger than that of *ctaA*, their patterns of induction and their dependencies on the ResD-ResE two-component regulators are strikingly similar in the wild-type strain.

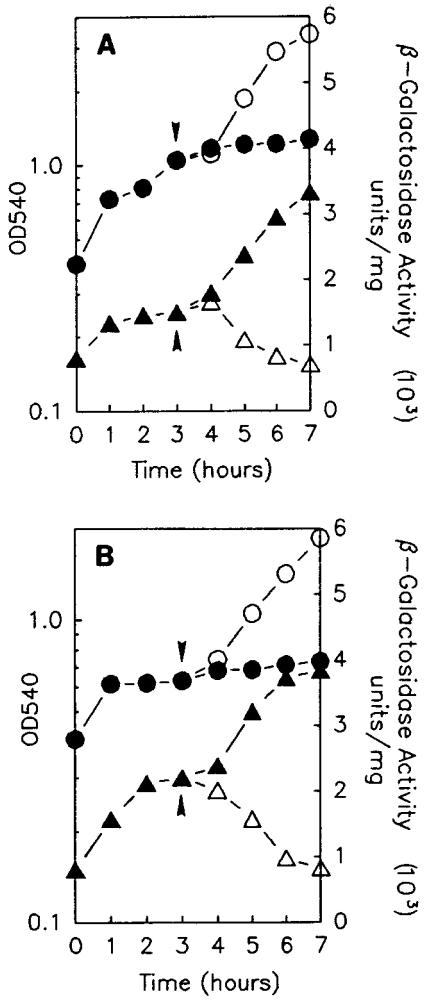


FIG. 5. Induction of the *resA* promoter by phosphate starvation or nitrogen deprivation. A late-exponential-phase culture of MH5201 (*resA-lacZ* in parent strain, JH642) was used to inoculate LPDM lacking phosphate (A) or ammonia (B). When growth slowed, because of a limiting nutrient, each culture was divided into two flasks and the limiting nutrient was added to one flask. (A) Growth and β-galactosidase activity of the culture starved for phosphate. The arrowhead indicates when the culture was divided and phosphate (final concentration, 1 mM) was added to one flask. (B) Growth and β-galactosidase activity of the culture starved for nitrogen. The arrowhead indicates the time when the culture was divided and ammonium sulfate (final concentration, 30 mM) was added to one flask. Symbols: ○, growth; △, β-galactosidase activity. Solid symbols represent starved cultures. Open symbols represent cultures supplemented with phosphate or nitrogen. OD540, optical density at 540 nm.

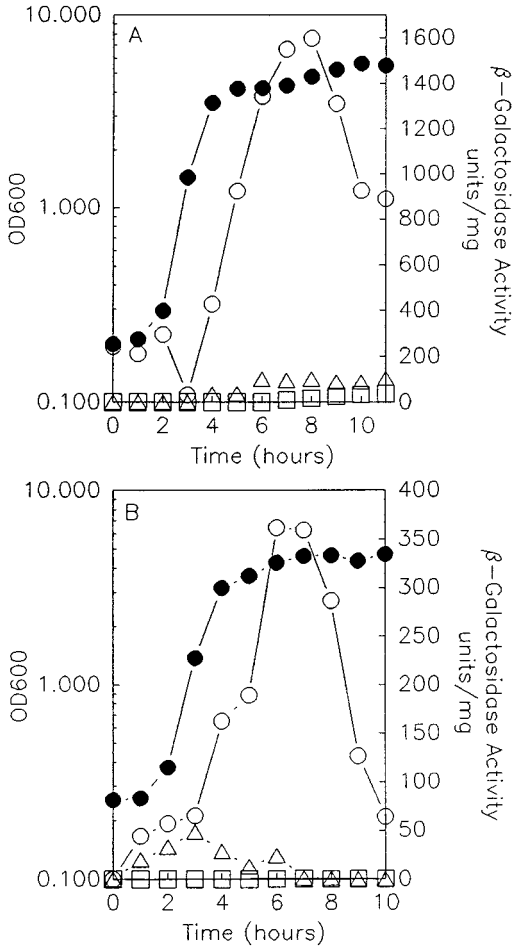


FIG. 6. Effect of mutations in *resD* or *resE* on the transcription of the *resA* operon and the *ctaA* gene. (A) Growth in SSG (solid symbols) for MH5201 (parent strain; growth was similar for MH5423 and MH5202) and β-galactosidase specific activity of *resA-lacZ* promoter fusion (open symbols) of MH5201 (parent strain) (○), MH5423 (*resE*) (△), and MH5202 (*resDE*) (□). (B) Growth in SSG (solid symbols) for MH5257 (parent strain; growth for MH5262 and MH5256 was similar) and β-galactosidase specific activity of the *ctaA-lacZ* promoter fusion (open symbols) of MH5257 (parent strain) (○), MH5456 (*resE*) (△), and MH5262 (*resDE*) (□). OD600, optical density at 600 nm.

(iv) *petCBD* operon. The genes which encode the respiratory cytochrome *bf* complex, which we called *petCBD*, were revealed during systematic sequencing of the *B. subtilis* chromosome region between the *trp* and *cotD* genes (48). PetC is similar to the Rieske-type iron-sulfur protein, and PetBI and PetD are similar to the αβ subunits of the cytochrome *b* dimer characteristic of cytochrome *bf* complexes (sequence similarity to be published elsewhere). We reasoned that the transcription of this operon might be dependent on ResD. (For reviews including the cytochrome *bf* complex, see references 59 and 60.) To identify the promoter responsible for transcription of the *pet* operon, RNA was isolated from cultures grown in minimal glucose medium or LB. Transcription of the cytochrome *bf* complex operon was initiated from the same apparent start site, 67 bp 5′ of the putative translation initiation site of *petC*. The transcript was not detected in RNA from cells harvested during mid-exponential growth in LB (Fig. 8A) but was easily identified in RNA from late-exponential-phase cells. Consensus sequences similar to those of a sigma A promoter were positioned at both −10 and −35 bp from the transcriptional start site (Fig. 8B).

That the transcription of the *pet* operon is highly induced at the end of exponential growth in LB was corroborated by Northern analysis. RNAs from the parent and *resDE* mutant (MH5081) strains were probed with a 1.2-kb DNA fragment which contained *petC*, *petB*, and an upstream gene. The 2.7-kb transcript of the *pet* operon was abundant in RNA isolated from late-exponential-phase cells of the parent strain but not in RNA from mid-exponential-phase cells and was not strongly induced at either stage in the *resDE* mutant (Fig. 9).

Phenotypes of strains with *resDE* transcription solely dependent on the internal promoter. Characterization of the *res* operon indicated a weak promoter positioned in front of *resDE* that functions primarily during exponential growth (Fig. 7). However, the major expression of this two-component system is from the *resA* promoter which activates transcription during postexponential growth (Fig. 6A). Analysis of MH5266, a strain with a Campbell insertion which separates transcription of *resABC* from that of *resDE*, allowed us to determine the phenotype of a strain for which transcription of *resDE* is solely dependent on the weak internal promoter. These data are summarized in Table 2 and show that the phenotypes of MH5266 differ from those of the *resDE* mutant, in that the former strain accumulates less acid when grown with glucose as a carbon source, is less streptomycin resistant, and can grow anaerobically.

Analysis of the contribution of reduced transcription of *resABC* to the *resDE* mutant phenotype. To determine the extent of the *resDE* mutant phenotype that results from reduced transcription of *resABC*, transcription of *resABC* was placed under control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter on plasmid pDH88. Campbell integration of the resulting plasmid, pES55, into the parent strain JH642 and the *resDE* mutant resulted in duplication of the three genes, one set controlled by the native promoter, the other by the P*spac* promoter of pDH88. The phenotypes of strain MH5451, the *resDE* mutant with *resABC* transcription under P*spac* control, are summarized in Table 2. IPTG induction of *resABC* resulted in increased streptomycin sensitivity and formation of a greater percentage of heat-resistant spores

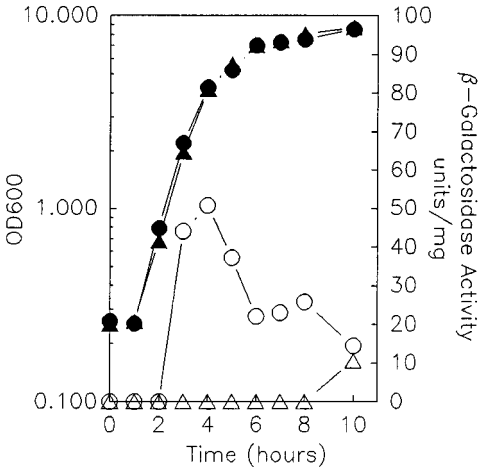


FIG. 7. Expression of the *resD* promoter. Growth in SSG (solid symbols) for MH5004 (JH642 with *resD-lacZ* fusion at *amyE* locus) and MH1000 (JH642 with promoterless *lacZ* at the *amyE* locus) and β-galactosidase specific activity of the *resD-lacZ* promoter fusion in MH5004 (○) and strain MH1000 (△) containing the promoterless *lacZ*.

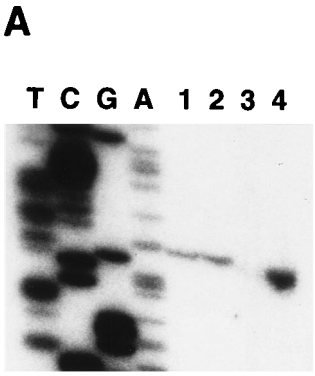


FIG. 8. Primer extension analysis of cytochrome *bf*-complex promoter. (A) Reverse transcription products were synthesized by annealing the end-labeled primer 5′ AGGCATCGACTTGATTAA 3′ to RNA isolated from mid (lanes 1 and 3)- and late (lanes 2 and 4)-exponential-phase cells grown in minimal glucose medium (lanes 1 and 2) or LB (lanes 3 and 4). The sequencing ladders indicate the sizes of the extended fragments, which equal 288 bases. (B) Promoter region sequence of cytochrome *bf* complex. MET, above ATG, marks the putative translation start site. The transcription start site, C, is double underlined, in boldface print, and marked with an asterisk. The putative −10 and −35 consensus sequences are underlined and in boldface print.

but had no effect on pigment formation, acid accumulation, TMPD oxidation, or anaerobic growth. The increase in streptomycin sensitivity without IPTG induction may indicate that the P*spac* promoter is not completely inactive when uninduced.

Strains were constructed to determine if induction of *resABC* from the P*spac* promoter either in the parental strain or in a *resDE* mutant would influence transcription of *ctaA-lacZ* or *resA-lacZ*. To construct MH5454 (*resA-lacZ* P*spac-resABC*), strain MH5450 (P*spac-resABC*) was made competent and transformed with MH5229 (*resA-lacZ* Sp^r) chromosomal DNA, selecting for spectinomycin resistance. To construct MH5459 (*ctaA-lacZ* P*spac-resABC*), strain MH5450 (P*spac-resABC* Cm^r) was transformed with pJL62, producing MH5455 (P*spac-res ABC* Sp^r), which was made competent and transformed with chromosomal DNA from MH5257, selecting for Cm^r.

To construct MH5458 (*resA-lacZ* P*spac-resABC resDE*), strain MH5454 was made competent and transformed with MH5081 chromosomal DNA, selecting for Tet^r. To construct MH5465 (*ctaA-lacZ* P*spac-resABC resDE*), strain MH5459 was made competent and transformed with MH5081 chromosomal DNA, selecting for Tet^r.

Strains MH5454, MH5459, MH5458, and MH5465 were grown in SSG with and without IPTG. β-Galactosidase expression from the *resA-lacZ* or *ctaA-lacZ* fusions was unchanged by IPTG induction of P*spac-resABC* in either the *resDE* mutant or the JH642 background (data not shown).

ResD is required for anaerobic growth and induction of respiratory nitrate reductase activity. The anaerobic growth of strains carrying mutations in *resDE* (MH5081) or *resE* (MH5418) was compared with that of the parental strain, JH642, in rich medium with and without added nitrate according to the procedure of Glaser et al. (11). After transfer to anaerobic conditions, the parental strain in medium supplemented with nitrate grew exponentially for several hours before reaching stationary phase; the same strain under the same conditions without nitrate failed to grow (Fig. 10A). The pattern of anaerobic growth of the *resDE* mutant, with or without nitrate, mimics the growth of the parental strain without nitrate, although the culture without added nitrate began lysing after 3 h (Fig. 10B). The *resE* mutant is capable of limited anaerobic growth with nitrate.

The level of nitrate reductase was measured in the three anaerobically grown cultures. The nitrate reductase production in the *resE* mutant strain MH5418 was drastically reduced compared with production in the parental strain, and the level of production in the *resDE* strain MH5081 was even lower (Fig. 11).

DISCUSSION

Our studies on the functions of *resD* and *resE* provide the first data concerning global regulation of respiration in an AT-rich gram-positive bacterium. The observation that the major transcript of the *res* operon includes *resD* and *resE* in addition to genes encoding proteins with similarity to those of cytochrome *c* biogenesis (ResA) (2) or a heme-binding motif (ResC) led us to explore the possibility that ResD and ResE play a regulatory role in respiration. The hypothesis was strengthened with the knowledge that *resD* and *ctaA* mutants share phenotypic characteristics. We showed that *resD* is required for expression of several components of the aerobic respiratory chain and for anaerobic growth on nitrate (Fig. 12). Mutations in *resD* severely decrease transcription from at least three promoters: *ctaA*, *petCBD*, and *resABCDE*. The absence of caa_3 and aa_3 terminal oxidases in a *resDE* mutant correlates well with the observation that a gene whose product is required

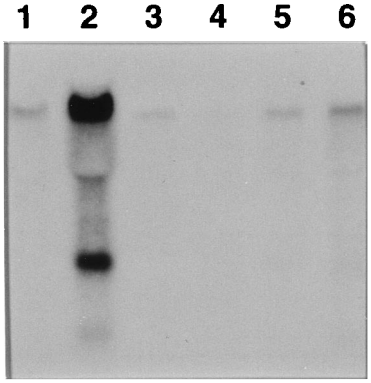


FIG. 9. Transcription of the cytochrome *bf* complex operon in the parent (JH642) and *resDE* mutant (MH5081) strains. RNA was isolated from JH642 (lanes 1, 2, and 3) and MH5081 (lanes 4, 5, and 6) cells at the middle (lanes 1 and 4) and end (lanes 2 and 5) of exponential phase and at early stationary phase (lanes 3 and 6) during growth in LB. A PCR-synthesized insert of an M13 phage, AGE2, containing the *petC* and *petB* genes, was used as a probe. The upper band (2.7 kb) corresponds to the cytochrome *bf* complex genes. The small band (1.2 kb) corresponds to the transcript upstream of *petC* (41a).

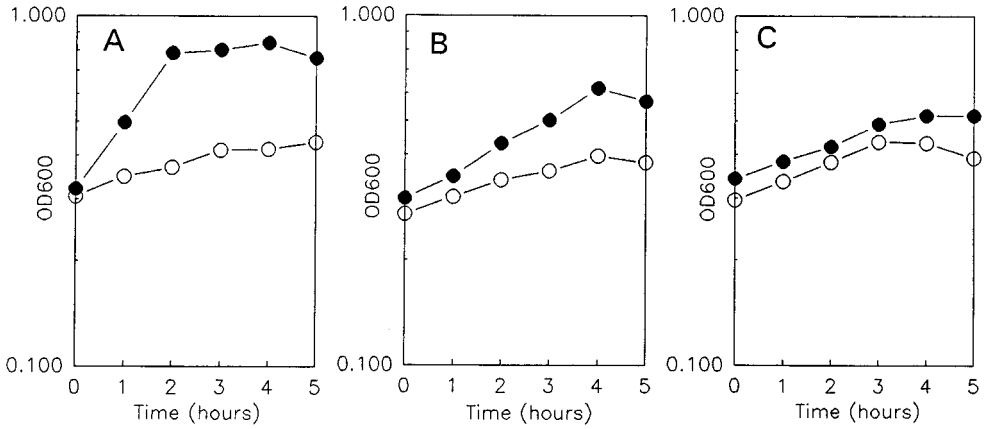


FIG. 10. Growth of *B. subtilis* under anaerobic conditions with (filled symbols) and without (open symbols) nitrate. (A) JH642 (parent strain); (B) MH5418 (*resE* mutant); (C) MH5081 (*resDE* mutant). OD600, optical density at 600 nm.

for the synthesis of heme A from heme O (57), *ctaA*, is not transcribed in the *resD* mutant. Further, *resDE* mutant phenotypes, including streptomycin resistance, inability to oxidize TMPD, and pink colony color and acid production during growth with glucose are consistent with those associated with cytochrome aa_3-deficient strains (34).

While some of the phenotypes of *resD* mutants mentioned above can be explained by a decrease in *ctaA* transcription, others cannot. The block of sporulation in these mutants may be different. *resD* mutants proceed as far as stage V, while *ctaA* mutants appear unable to initiate sporulation. Another phenotype not shared with a *ctaA* mutant is the inability of a *resD* mutant to grow anaerobically. The *ctaA* mutant grows as well as its parent strain under anaerobic conditions (55), indicating that the inability of the *resD* mutant to grow anaerobically is due to a regulatory role in respiration separate from heme A biosynthesis, possibly an effect on the transcription of genes encoding respiratory nitrate reductase, *narGHJI* (Fig. 12). Levels of nitrate reductase produced in a *resDE* mutant during anaerobic growth were drastically reduced compared with wild-type levels. Other genes such as *narA*, which is implicated in the biosynthesis of a molybdenum cofactor required for both nitrate assimilation and respiration, and *fnr*, which is also required for anaerobic nitrate reductase activity (37), are also candidates for *resDE* regulation.

How extensive is the regulation of ResD (and to a lesser extent ResE) in respiration? We have shown that ResD is not essential for transcription of genes encoding early heme biosynthesis in the *hemA* operon (19). It seems possible that null *resD* mutants fail to grow on nonfermentable carbon sources because decreased capacity for respiration results in an accumulation of NADH, thereby limiting the availability of NAD for other cellular functions. Alternatively, if ResD is also required for expression of ATPase subunit genes, the generation of ATP by substrate-level phosphorylation would be essential for growth of *resD* mutant strains.

It is important that in no case, either in regulation of genes involved in aerobic respiration or in regulation of genes involved in anaerobic respiration, is it established that the activation of expression of any gene by ResD is direct. The signal transduction pathway controlling respiration surely includes a number of regulatory proteins, activators or repressors of transcription, or even other two-component systems, which must be identified before the signal transduction circuitry can be pieced together.

We have shown that there are parallel pathways for activation of the *phoPR* operon encoding Pho regulon regulators, PhoP and PhoR (22, 54) and that ResD is required for one of the pathways. We believe that ResD regulation of the Pho regulators is indirect (17). Compensatory mutations in the *resDE* mutant background restore Pho regulation to wild-type levels. Interestingly, these same compensatory mutations restore transcription of the *resA* promoter but not that of the *ctaA* promoter (17, 55). These data suggest that the autoinduction of the *resABCDE* operon by ResD is indirect but leave open the possibility that ResD directly regulates *ctaA* transcription.

We have previously noted preliminary data in a review (16) concerning phenotypes of the *resE* mutant strain MH5061 which are less severe than those of the *resE* mutant strain MH5418 reported here. The original *resE* mutation (16) was made by Campbell integration of a plasmid carrying an internal fragment of the *resE* gene which may have fortuitously produced a truncated protein with partial activity. The *resE* mutant used in these studies contains a major deletion within

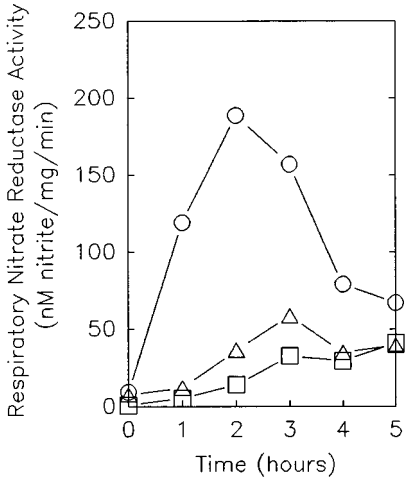


FIG. 11. Nitrate reductase activity in *res* mutants and parent strains. Respiratory nitrate reductase was measured as described in Materials and Methods by using cells transferred to anaerobic conditions at hour 0. ○, wild type; △, *resE* mutant; □, *resDE* mutant.

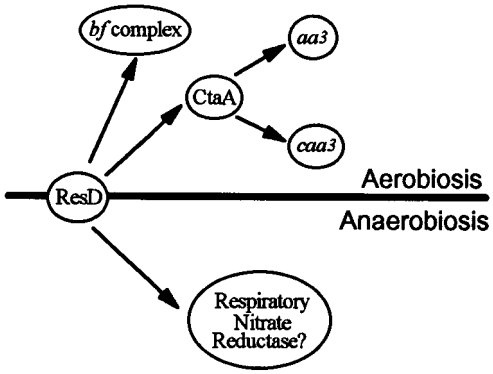


FIG. 12. Model for the regulation of respiration by ResD. Arrows indicate positive regulation.

the gene into which a resistance gene was cloned. Even in the deletion strain MH5418, the *resE* mutant phenotype is not as deleterious as a *resD* mutation. We offer three explanations for the fact that mutations in *resE* are less deleterious to the cell than a *resD* mutation or a *resDE* double mutation. First, ResD may not require phosphorylation for activity, at least for the activation of respiratory gene transcription. Another *Bacillus* response regulator, DegU, is active when it is not phosphorylated and has a different function when it is (33); this may be the case for ResD also. Second, ResE may be only one of several sensor kinases that interact with ResD. ResD may mediate an adaptive response required under several stress conditions, and different sensor kinases may be necessary to activate it under each of these. There are examples of multiple sensor kinases acting independently to activate a response regulator. Two sensor transmitters, NarX and NarQ, are implicated in nitrate detection and can function independently to activate NarL, the response regulator activating nitrate-dependent transcription of genes for anaerobic electron transport in *E. coli* (12, 44). The two histidine kinases may have different roles, since NarL is phosphorylated and phosphatased at different rates by NarX and NarQ. Evidence for multiple histidine kinases indirectly activating the same response regulator comes from the signal transduction pathway necessary for the initiation of *B. subtilis* sporulation, the phosphorelay (5). If multiple sensor kinases capable of activating ResD exist, we have shown that PhoR, DegS, and ComP are not among them (21). Finally, ResD may be phosphorylated by low-molecular-weight phospho-donors such as acetyl phosphate or phosphoramidate in the absence of ResE (31, 32).

The fact that ResD is required for transcription from the *resA* promoter raises two questions. First, since *resD* is apparently part of the *resA* operon, how is it expressed independently in order to participate in transcription of the operon? The answer evidently is that *resD* is transcribed from its own promoter at low levels during exponential growth and, thus, is available at the beginning of stationary phase when transcription from the *resA* promoter increases markedly. This low-level transcription of *resDE* is also critical for providing adequate ResD to activate low levels of *ctaA* and *resA* operon transcription during vegetative growth in wild-type strains, transcription that is absent in the *resD* mutant (Fig. 12). Since this regulatory system exhibits a positive-feedback property, there should also be a negative regulatory control, perhaps governing the phosphorylation of ResD or perhaps via the products of genes in which compensatory mutations were isolated.

Second, given the fact that *resD*, perhaps with *resE*, is required for transcription from the *resA* promoter, why are mutations in the first three open reading frames of the *res* operon (*resA*, *resB*, and *resC*) lethal to the cell, while those in *resD* or *resE* are not? Perhaps low levels of transcription from *resA* in the absence of *resD* is sufficient for survival of the cell though not for normal function. Another explanation is that there may be some read-through transcription from the orfX13 promoter, since there is no obvious transcriptional terminator between orfX13 and *resA*. Indeed, a minor transcript long enough to include orfX13 through orfX18 (*resE*) was detected (1) by Northern analysis with probes from either region.

Further studies are required to determine the functions of the apparently essential genes in the *res* operon. Information concerning the functions of ResA, ResB, and ResC derived from sequence analysis suggests that they function in respiration, perhaps in cytochrome *c* biogenesis. IPTG induction of these genes in a *resDE* background increased the streptomycin sensitivity of the strain and the number of heat-resistant spores produced under sporulation conditions. Expression of these genes did not bypass the ResDE requirement for anaerobic growth or oxidation of the artificial electron donor TMPD, nor did it reverse the red pigment or acid accumulation of colonies containing mutations in *resDE*.

Our studies of respiration regulation in *B. subtilis* by ResD suggest that the regulatory mechanism is quite different from that in *E. coli*, as indicated by current studies of ArcA, NarL, NarP, and Fnr. In *E. coli*, genes involved in aerobic respiration, including the *cyo* operon, which encodes cytochrome *o* oxidase, are regulated by repression via ArcA and Fnr (24, 50). ArcA and Fnr play positive roles in anaerobic respiration. Dual signal transduction systems comprising NarL, NarP, NarQ, and NarX are involved in nitrate regulation of anaerobic respiration in which formate is an electron donor for nitrate reduction (53). Our data show that ResD plays a positive role in affecting both aerobic and anaerobic respiration in *B. subtilis*.

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