Virulence Gene Expression Is Independent of ResDE-Regulated Respiration Control in *Bacillus anthracis*

Adam C. Wilson, James A. Hoch, and Marta Perego*

Division of Cellular Biology, Department of Molecular and Experimental Medicine, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

Received 29 February 2008/Accepted 25 May 2008

The ResDE two-component system regulates the synthesis of several components of the aerobic and anaerobic respiratory pathways in bacilli. The ResD response regulator transcription factor has been implicated in the regulation of virulence factors in a number of gram-positive species, including *Bacillus anthracis*. The precise deletions of *resD* and *resE* in *B. anthracis* that retained the classical respiratory phenotypes did not affect the expression of the gene for the protective antigen of the anthrax toxin, *pagA*, or that of the toxin regulator, *atxA*. The results indicate that the loss of ResDE-controlled respiratory capacity does not affect the synthesis of anthrax toxin.

*Bacillus anthracis* is a gram-positive, facultatively anaerobic, endospore-forming bacterium that is the etiological agent of anthrax. Full virulence in the host requires the presence of both the anthrax toxin and capsule, each encoded by genes carried by separate virulence plasmids (16). The expression of toxin and capsule genes is influenced by a number of environmental and growth conditions, with maximal expression occurring at 37°C in the presence of CO₂ bicarbonate (14, 15). The global virulence regulator AtxA is essential for expression of both the toxin and capsule (4, 5, 11, 24, 25). However, the mechanisms controlling the virulence regulatory circuit in *B. anthracis* are incompletely understood, as regulation through AtxA alone cannot account for all regulatory inputs known to influence toxin and capsule gene expression.

Two-component signal transduction systems are a common mechanism by which bacteria regulate gene expression in response to environmental inputs (9). One such two-component system, ResD/ResE, had long been known to regulate respiratory pathways in *Bacillus subtilis* (22). Orthologous ResD/ResE systems have been shown to regulate virulence gene expression in *Staphylococcus aureus* and *Listeria monocytogenes* (12, 27), as well as endotoxin production in *Bacillus cereus*, although only under anaerobiosis (6).

Recently, Vetter and Schlievert reported the generation and characterization of a *B. anthracis* *resD* mutant strain (which is most likely a *resDE* double mutant, based on the oligonucleotide primers used by these authors to generate the mutation) and suggested that the ResDE system is required for the active expression of toxin genes and the virulence regulator *atxA* (26).

In the *B. anthracis* Sterne strain, the ResDE two-component signal transduction system is encoded by the BAS1386 and BAS1387 genes. BAS1386 (GenBank ID AAT53706) shares 78% amino acid identity with the *B. subtilis* ResD response regulator, while BAS1387 (GenBank ID AAT53707) has 52% amino acid identity with the *B. subtilis* ResE sensor histidine kinase. In *B. anthracis*, the *resABCDE* locus is organized similarly to that in *B. subtilis*, with the respiratory genes *resABC* located upstream of *resDE* (22). Recent findings in this laboratory suggest that the *B. anthracis* ResDE-regulated genes *resBC*, carried in an operon with *resDE*, contribute to virulence gene expression in an *atxA*-dependent manner (A. C. Wilson, J. A. Hoch, and M. Perego, unpublished data).

To investigate the role of ResDE in virulence gene expression, we generated markerless *resD*, *resE*, and *resDE* deletion strains in a *B. anthracis* 34F2 (pXO1” pXO2”) background. A 475-bp region upstream of BAS1386 was PCR amplified using primers oresDU5’ Bam and oresDU3’ Sal, while a 768-bp region downstream of BAS1386 was amplified using primers oresDD5’ Sal and oresDD3’ (see Table S1 in the supplemental material) and Accu enzyme DNA polymerase (Bioline). The upstream PCR fragment was cloned into the BamHI and SalI restriction sites of pORI-I-SceI to generate plasmid pAW074. The downstream region was then cloned into the SalI and PstI sites of pAW074 to generate pAW075.

For the deletion of *resE*, the upstream fragment was generated with oligonucleotide primers resEU5’ Bam and resEU3’ Sal, while the downstream fragment was obtained with primers resEDS5’ Sal and resED3’ Pst. Cloning of these fragments in pORI-I-SceI resulted in plasmid pAW140. The deletion of *resDE* was obtained by cloning in pAW074 the fragment obtained by PCR amplification with oligonucleotide primers resEDS5’ Sal and resED3’ Pst, giving rise to plasmid pAW138.

Plasmids pAW075, pAW140, and pAW138 were electroporated into *B. anthracis* 34F2 (11), and the protocol of Janes and Stibitz (10) was used to generate the markerless deletions. Diagnostic PCR was carried out to ensure that the entire coding sequences had been correctly deleted (see Fig. S1 in the supplemental material) and that the pXO1 plasmid did not get lost in the process. All growth media for the deletion strains after electroporation contained 0.2% glucose to reduce aero-
bic respiration and prevent spontaneous generation of compensatory mutations (20).

Deletion of resD in B. anthracis resulted in a number of phenotypic effects, which are summarized in Table 1. The resD mutant grew poorly compared to the parental strain on complex media (LB medium with or without 0.2% glucose) (Fig. 1A; see Fig. S2 in the supplemental material) or defined, toxin-inducing medium (R medium, which contains 0.25% glucose and 0.8% sodium bicarbonate in a 5% CO$_2$ atmosphere) (18) (Fig. 2A), consistent with the phenotypes of B. subtilis with the same mutation (22). Notably, the addition of 0.2% glucose to LB medium and the increase of glucose in the R medium to 0.54% improved cell growth but still did not fully restore the growth rate of the parent (Table 1; and data not shown). The resD mutant was largely incapable of growth on nutrient sporulation medium phosphate (NSMP) (8) most likely due to the repression exerted by the phosphate in the medium on the global virulence regulator AtxA. The resE component of the tripartite anthrax toxin, while oxidize the artificial electron donor TMPD (N,N,N’N’-tетраметил-p-фениленедиамин) (13), indicating the lack of a-type terminal oxidases and a disruption of aerobic respiration, thus confirming the deletion of the resD gene (see Fig. S3 in the supplemental material).

The phenotypes of the resDE double mutant were essentially identical to the ones described for the resD single mutant. As expected on the basis of studies on the resDE system of B. subtilis (22), the deletion of the resE gene encoding the histidine sensor kinase resulted in less-dramatic phenotypes: the TMPD activity was only partially affected, sporulation was similar to that of the parental strain, and growth in LB broth, with or without glucose, was not affected (see Fig. S4 in the supplemental material; and data not shown).

The effect of resD deletion on the expression of virulence genes was assayed by monitoring β-galactosidase expression of a pagA-lacZ or an atxA-lacZ reporter on the replicative vector pTCV-lac (17), pagA encodes the protective antigen (PA), a component of the tripartite anthrax toxin, while atxA encodes the global virulence regulator AtxA. The resD strain and the parental 34F2 strain were transformed by electroporation with pTCV-lacpagA (23) and pTCVlac-ataxA2 (3) to generate virulence reporter strains. Interestingly, the resD strain containing either of the LacZ reporter plasmids was unable to grow in

![FIG. 1. Cell growth and virulence factor expression in B. anthracis 34F2 parental and resD deletion strains. Cells carrying a pagA-lacZ or atxA-lacZ fusion were grown in LB medium supplemented with kanamycin. β-Galactosidase assays were carried out on samples taken at hourly intervals as indicated. (A) Cell growth of pagA-lacZ strain (growth was similar for atxA-lacZ strains); (B) pagA-lacZ expression; (C) atxA-lacZ expression. Symbols in all three panels: •, parental strain; ×, resD mutant; ■, resDcmp1 mutant; ▲, resDcmp2 mutant.](http://jb.asm.org/Downloaded from http://jb.asm.org/ August 15, 2017 by guest)
the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) but could grow in the presence of either the LacZ-carrying plasmid or X-Gal alone, suggesting a strong sensitivity to the insoluble 5,5′-dibromo-4,4′-dichloro-indigo product of X-Gal cleavage by β-galactosidase. β-Galactosidase assays carried out on parental and resD mutant strains were performed as previously described (7). Cells were lysed with a buffer containing 600 μg/ml lysozyme, 0.6% Triton X, and 1 unit of mutanolysin (Sigma).

When parental and resD deletion strains were grown in LB broth without glucose, no significant difference in normalized pagA-lacZ (Fig. 1B) or atxA-lacZ (Fig. 1C) expression levels were observed. Similarly, the addition of 0.2% glucose to LB media, thereby reducing the requirement for terminal oxidases, did not alter pagA-lacZ or atxA-lacZ expression in the mutant relative to the wild-type strain but improved the growth rate of the mutant (data not shown). Also, there was no difference between parental and resD deletion strains in either pagA-lacZ (Fig. 2B) or atxA-lacZ (Fig. 2C) expression when cells were grown in toxin-inducing R medium in a 5% CO₂ atmosphere.

Essentially the same results were obtained when the pagA-lacZ and atxA-lacZ reporters were assayed in the resDE and resE mutant strains (see Fig. S4 in the supplemental material; and data not shown).

In order to confirm these results, we quantitated the levels of PA and AtxA protein in the supernatant and cell lysate, respectively, of the parental and resD mutant strains. The results, shown in Fig. 3, confirmed that the resD deletion did not affect PA and AtxA protein levels.

Our observations are inconsistent with those of Vetter and Schlievert (26), who found a substantial decrease in pagA and atxA expression levels and, surprisingly, no effect on the aerobic growth rate of a resD mutant (26). The clear growth defect originally observed in B. subtilis resD deletion strains (22) was also found in resD strains of Listeria monocytogenes (12) and in a B. cereus food-borne pathogenic strain (6). One explanation for the difference in these findings from those of Vetter and Schlievert (26) may stem from the propensity of resD deletion strains in all species to accumulate compensatory mutations that spontaneously appear as faster-growing opaque papillae when resD strains are grown on media without glucose. In B. subtilis, resD deletion strains spontaneously develop compensatory mutations, some of which upregulate the expression of cytochrome bd, an alternative terminal oxidase not requiring expression of the ResD-regulated ctaA gene (20–22).

To investigate the possibility that such spontaneous compensatory mutations may influence virulence gene expression, the B. anthracis resD strain, which had previously been maintained on media containing 0.2% glucose, was grown on TBAB plates without glucose, and two faster-growing opaque papillae were isolated. The phenotypes of the mutants were analyzed; they grew more robustly on TBAB, were able to grow on NSMP without glucose, oxidized TMPD, and showed partial restoration of sporulation when grown on SM (Table 1). The two compensatory mutants (named resDcmp1 and resDcmp2) were

FIG. 2. Cell growth and virulence factor expression in B. anthracis 34F2 parental and resD deletion strains. Cells carrying a pagA-lacZ or atxA-lacZ fusion were grown in R medium (containing 0.25% glucose) supplemented with kanamycin in a 5% CO₂ atmosphere, β-Galactosidase assays were carried out on samples taken at hourly intervals as indicated. (A) Cell growth of atxA-lacZ expression levels and, surprisingly, no effect on the aerobic growth rate of a resD mutant (26). The clear growth defect originally observed in B. subtilis resD deletion strains (22) was also found in resD strains of Listeria monocytogenes (12) and in a B. cereus food-borne pathogenic strain (6). One explanation for the difference in these findings from those of Vetter and Schlievert (26) may stem from the propensity of resD deletion strains in all species to accumulate compensatory mutations that spontaneously appear as faster-growing opaque papillae when resD strains are grown on media without glucose. In B. subtilis, resD deletion strains spontaneously develop compensatory mutations, some of which upregulate the expression of cytochrome bd, an alternative terminal oxidase not requiring expression of the ResD-regulated ctaA gene (20–22).

To investigate the possibility that such spontaneous compensatory mutations may influence virulence gene expression, the B. anthracis resD strain, which had previously been maintained on media containing 0.2% glucose, was grown on TBAB plates without glucose, and two faster-growing opaque papillae were isolated. The phenotypes of the mutants were analyzed; they grew more robustly on TBAB, were able to grow on NSMP without glucose, oxidized TMPD, and showed partial restoration of sporulation when grown on SM (Table 1). The two compensatory mutants (named resDcmp1 and resDcmp2) were

FIG. 3. Toxin and AtxA protein levels in B. anthracis 34F2 parental strain and isogenic resD deletion mutants. (A) Western blot analysis carried out on B. anthracis culture supernatants collected from two independent cultures grown in R medium in a 5% CO₂ atmosphere and detected with polyclonal anti-PA antibody. Lanes: 1, MagicMarker XP (Invitrogen); 2, parental strain; 3, parental strain 2; 4, resD 1 mutant; 5, resD 2 mutant. (B) Western blot analysis carried out on B. anthracis cell lysates collected from the same two independent cultures used for panel A and detected with polyclonal anti-AtxA antibody. Lanes: 1, purified AtxA; 2, MagicMarker XP; 3, parental strain 1; 4, parental strain 2; 5, resD culture 1; 6, resD culture 2. Sample preparation, antibody production, and Western blot analyses were carried out as described by Tsvetanova et al. (23). Samples were collected after 9 h of growth and normalized to an optical density at 600 nm of 1.5.
transformed with pTCVlac-pagA and pTCVlac-atxA12, and assayed for β-galactosidase activity. Both compensatory mutants showed increased growth rates on LB medium (Fig. 1A) and R medium (Fig. 2A) relative to the resD deletion strain. However, neither compensatory mutant showed a significant alteration in pagA or atxA expression in either LB or R medium (Fig. 1B and C and 2B and C).

Our observations indicate that the ResD/ResE two-component signal transduction system does not regulate, directly or indirectly, toxin gene expression in B. anthracis under aerobic growth conditions. As in B. subtilis, deletion of B. anthracis resD results not only in a growth defect under aerobic growth conditions but also in a complex phenotype resulting from disruption of respiratory pathways. The altered regulation of respiration may indirectly affect pathogenesis; however, in our study, deletion of genes encoding the ResD transcription factor did not affect the transcription of either the PA gene or the master virulence regulator AtxA-encoding gene. Toxic gene expression was also not affected by the deletion of the resE gene alone, thus distinguishing B. anthracis from B. cereus, in which the absence of the ResE histidine kinase was shown to suppress almost completely the production of enterotoxin (6).

The double deletion of resD and resE also did not affect toxin gene expression.

resD deletion strains are difficult to characterize and maintain due to their instability and propensity to develop compensatory mutations. The number and variety of compensatory mutants induced simply by growing a resD strain on media without glucose raise the possibility of alternative mutations that can decrease virulence factor gene expression, though neither of the compensatory resD mutants analyzed in this study affected pagA or atxA expression. Investigators should be aware of the problem of resD strains when assigning phenotypes or functions to these strains.

This research was supported by grant AI055860 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Oligonucleotide synthesis and DNA sequencing were sponsored in part by the Stein Beneficial Trust.

This is manuscript 19422 from The Scripps Research Institute.

REFERENCES