NOTES

Dual Control of *sbo-alb* Operon Expression by the Spo0 and ResDE Systems of Signal Transduction under Anaerobic Conditions in *Bacillus subtilis*

MICHIKO M. NAKANO, GUOLU ZHENG, AND PETER ZUBER*

Biochemistry and Molecular Biology, Oregon Graduate Institute of Science and Technology, Beaverton, Oregon 97006

The *Bacillus subtilis* *sbo-alb* operon contains *sboA*, the structural gene for the bacteriocin subtilosin, and the *alb* genes required for subtilosin production. Transcription from the *sbo-alb* promoter is highly induced by oxygen limitation. The transcriptional regulation of the *sbo-alb* operon is under dual control involving the transition state regulator AbrB and the two-component regulatory proteins ResD and ResE.

The production of bacteriocins is usually induced by a combination of high cell density and low nutrient availability (12, 13, 19). The production of antimicrobial compounds will allow the bacterium to compete for scarce sources of carbon, nitrogen, and energy. Bacteriocins are often produced at high cell density to ensure that a concentration of antibiotic high enough to have an impact on the local environment is achieved. The spore-forming soil bacterium *Bacillus subtilis* produces an assortment of antimicrobial compounds, including the antilisterial bacteriocin subtilosin (1, 36). The structural gene for subtilosin, *sboA*, resides at the 5′ end of an operon that contains the *alb* genes (14, 34; T. Stein, S. Düsterhaus, A. Stroh, and K.-D. Entian, Abstr. 10th Int. Conf. Bacilli, abstr. P103, p. 65, 1999), which are believed to function in subtilosin chemical modification, processing, and export. The regulation of *sbo-alb* operon expression is complex, since it is induced in late growth cultures apparently in response to starvation and is also dramatically induced by oxygen limitation (29, 34; Stein et al., Abstr. 10th Int. Conf. Bacilli). Many of the factors governing gene expression in response to starvation (6, 8, 20, 28) and oxygen limitation (4, 25, 26, 31) have been identified in *B. subtilis*. Several genes that are normally expressed in response to nutritional stress are subject to repression by the transition state regulatory protein AbrB (29). The product of the *abrB* gene binds directly to promoter DNA of the genes that are induced by starvation and prevents their transcription during robust culture growth. We have found that *sbo-alb* is one of the operons that are negatively controlled by AbrB (34). The *abrB* gene is negatively controlled by the key transcriptional regulator of starvation-induced genes Spo0A (7, 30).

While several studies of the control of bacteriocin production in response to high cell density and nutritional stress have been reported, there are few that have uncovered induction of bacteriocin synthesis in response to anaerobiosis. Colicin E1 produced by *Escherichia coli* is the product of the *cea* gene that is transcriptionally induced under anaerobic conditions (5, 18). Activation of *cea* transcription requires the FNR protein, an iron-binding transcriptional activator of many anaerobically induced genes (5, 18).

In this paper, we report that anaerobic induction of the *sboA-alb* genes is only conditionally dependent on FNR but absolutely requires the ResDE signal transduction complex. An examination of the epistatic relationship of the various genes that function in *sbo-alb* control shows that both the Spo0-AbrB and ResDE systems function independently in the control of *sbo-alb* transcription.

Transcription of *sbo-alb* is induced by oxygen limitation. The *sbo-alb* operon contains nine genes (*sboAX-albABC-DEFG*) and is transcribed from a σ^A-type promoter residing upstream of the *sboA* gene (34). Although transcription proceeds through the *alb* genes, there exists a sequence overlapping the end of *sboA* and internal to the *sboX* coding sequence that can potentially form a stable hairpin-loop structure which would be expected to impede transcription from the *sbo-alb* promoter. To study the regulation of *sbo-alb* expression, two *lacZ* transcriptional fusions were constructed as previously described (34). One fusion, *sboΔBH-lacZ*, carries a fragment of the 5′ half of the *sboA* gene, along with its promoter region, upstream of a promoterless *lacZ* gene that bears a *B. subtilis* ribosome-binding site. The plasmid containing the *sboΔBH-lacZ* fusion was integrated into an SPβ specialized transducing phage. The other *lacZ* fusion was constructed by inserting a fragment containing a segment of the *sbo-alb* operon from the middle of *albA* to the middle of *albC* into the integrative plasmid pMUTIN2 (33), thereby placing the fragment upstream of the promoterless *lacZ* gene. Integration of this plasmid, pMUPE1 (34), into the *sbo-alb* operon creates an (*sbo-alb*)-*lacZ* transcriptional fusion that does not disrupt any of the open reading frames within the operon but does disrupt the *sbo-alb* transcription unit (34).

The fusion-bearing derivatives of strain JH642 were grown aerobically and anaerobically in 2xYT medium (23) supplemented with 1% glucose and 0.2% KNO_3 as previously described (22). Both fusions were poorly expressed in aerobic cultures (Fig. 1) as previously shown (34), but a dramatic, 600-fold induction of phage-borne *sboΔBH-lacZ* was observed in anaerobically grown cultures. The *alb*::pMUPE1 fusion was also induced 40-fold late in the growth curve under anaerobic conditions.

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Biology, Oregon Graduate Institute of Science and Technology, 20000 NW Walker Rd., Beaverton, OR 97006. Phone: (503) 748-7335. Fax: (503) 748-1464. E-mail: pzuber@bmb.ogi.edu.
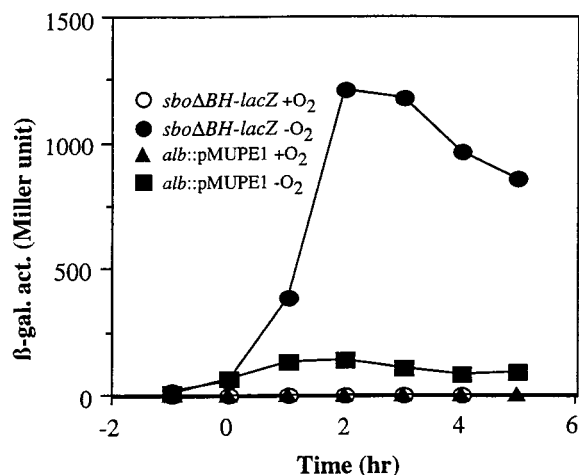
FIG. 1. Anaerobic expression of *sbo-alb* and subtilosin production. Expression of *sboA-lacZ* and *alb-lacZ* fusions in strains ORB3147 (*alb*::pMUPE1) and ORB3162 (SPβ*sboΔBH-lacZ*) under aerobic (+O₂) and anaerobic (−O₂) growth conditions. β-gal. act., β-galactosidase activity.

TABLE 1. Expression of *sboΔBH-lacZ*

Strain	Relevant genotype	β-Galactosidase activity (Miller units)[a]	
		Nitrate	Nitrite
ORB3162	Wild type	1,462	2,096
ORB3349	*fnr::spc*	42	2,182
ORB3350	*ΔnarGH::phleo*	52	1,777
ORB3348	*ΔresDE::tet*	7	13
ORB3437	*spo0A::erm*	220	391
ORB3241	*abrB::neo*	1,681	NT[b]
ORB3438	*spo0A::erm abrB::neo*	2,042	NT
ORB3384	*ΔresDE::tet abrB::neo*	14	NT

[a] Cells were grown anaerobically in 2xYT medium with 1% glucose supplemented with 0.2% KNO_3 or 10 mM KNO_2. The maximal activity of at least two independent experiments is shown.
[b] NT, not tested.

The accelerated expression of *sbo-alb* in anaerobically grown cells prompted us to determine if transcription from the *sbo-alb* promoter is stimulated under oxygen limitation and if the same promoter is utilized in anaerobic growth as in aerobic growth. Total RNA was purified, as described in a previous report (24), from JH642 cells collected at T_2 of the growth curve (2 h after the end of exponential phase). Cultures were grown aerobically and anaerobically at 37°C in 2 × YT medium supplemented with 1% glucose and 0.2% KNO_3. Primer extension was performed as previously described using oligonucleotide osboP4 (34). An at least 10-fold greater amount of primer extension product was produced in reaction mixtures containing RNA from anaerobically grown cells (Fig. 2A, lane 6) than in reaction mixtures containing RNA from aerobically grown cells (lane 5). It was also observed that the same transcriptional start site was utilized under both aerobic and anaerobic conditions.

Anaerobic induction of *sbo-alb* requires the ResDE signal transduction system. The involvement of the known regulators of anaerobic gene control was next examined by measuring the expression of *sboΔBH-lacZ* in *resDE* (26, 31), *fnr* (4, 26), and *narGH* (21) mutant cells. The *narGHJ* genes encode subunits of respiratory nitrate reductase (9, 15) and are required for the expression of some anaerobically induced genes in the absence of nitrite (15, 26). These genes also require *fnr*, since *narGHJI* transcription is activated by the FNR protein (15, 22, 26). As shown in Table 1, all three mutations abolish *sboΔBH-lacZ* expression in 2xYT-glucose-nitrate-grown cells under anaerobic conditions. If nitrite (10 mM KNO_2) is substituted for nitrate, then expression of *sboΔBH-lacZ* no longer requires *fnr* and *narGH* but still is absolutely dependent on the ResDE system (Table 1).

Transcription of *sbo-alb* is controlled by two independent regulatory pathways. The transition state regulator AbrB exerts negative control over transcription from the *sbo-alb* promoter (34). This form of regulation was reexamined in anaerobically grown cells bearing the *sboΔBH-lacZ* fusion. A mutation in the *spo0A* gene (10) which results in overproduction of the *abrB* product causes a sharp reduction in *sboΔBH-lacZ* expression in cells grown anaerobically (Table 1). The
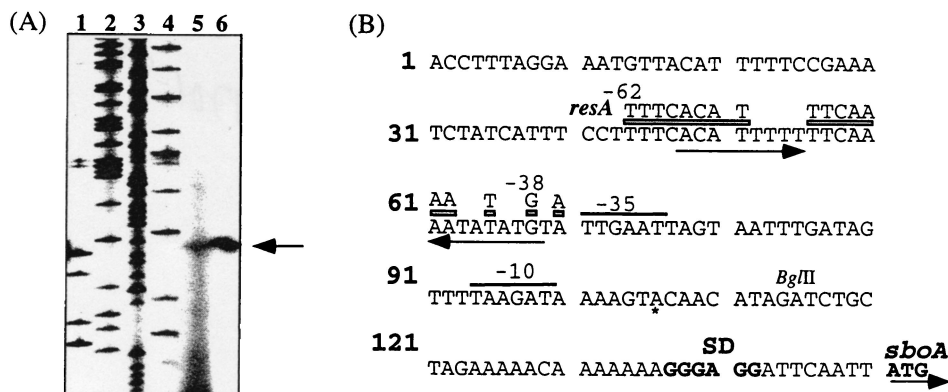

FIG. 2. (A) Utilization of the same transcription start site in the *sbo-alb* promoter region under aerobic and anaerobic conditions. Tenfold less primer extension reaction mixture was applied to lane 6 (RNA from anaerobic cultures) than was applied to lane 5 (RNA from aerobic cultures). Sequencing reactions G, A, T, and C are in lanes 1, 2, 3, and 4, respectively. (B) Nucleotide sequence of the *sbo-alb* promoter region and similarity to the regulatory region of the *resA* operon. The ATG start site of the *sboA* gene and its ribosome-binding site (SD) are shown. The −10 and −35 regions are indicated, as is the transcription start site (∗), and a region of approximate dyad symmetry upstream of the −35 sequence is marked with arrows beneath the nucleotide sequence. A broken bar above the nucleotide sequence extending from −36 to −62 marks the sequence identity between the *resA* and *sbo-alb* regulatory regions. The sequence above the bar indicates the nucleotide residues of the corresponding region in the *resA* promoter region.

effect of a *spo0A* mutation is overcome by introducing a mutation in *abrB* (34) (strain ORB3438, Table 1). Since the temporal regulation of *sbo-alb* expression is still observed in the *abrB* and *spo0A abrB* mutants (data not shown), ResD might be activated only after exponential growth, probably as a result of nitrite accumulation. However, we cannot rule out the possibility that another regulator is involved in the postexponential induction of *sboA-alb*.

ResDE, like the Spo0 system, could positively regulate *sbo-alb* transcription by repressing the *abrB* gene or indirectly affect AbrB concentration and/or activity. If this were the case, then we would expect an *abrB* null mutation to result in *sbo-alb* expression in the absence of ResDE. Examination of *sboΔBH-lacZ* expression in the *resDE abrB* double mutant showed that the ResDE system is still required for *sbo-alb* transcription in the absence of AbrB protein (Table 1).

To further investigate the relationship among Spo0A, AbrB, and ResDE, the expression of ResDE-controlled genes was examined in *spo0A*, *abrB*, and *spo0A abrB* mutant cells bearing either an *fnr* (26)-, an *hmp* (flavohemoglobin) (15)-, or an *nasD* (nitrite reductase) (22)-*lacZ* construct, each of which requires ResDE for expression. If expression of *resDE* or the activity of their products required *spo0A* or was repressed by AbrB, then a *spo0A* mutation would have resulted in reduced expression of the *resDE*-controlled *lacZ* fusions. No effect of a *spo0A* or an *abrB* mutation on *lacZ* expression was detected (data not shown), indicating that Spo0A and AbrB do not influence the activity of ResDE in anaerobically grown cells. The addition of nitrite, which is thought to be necessary for ResDE-dependent activation of anaerobically induced genes, did not significantly overcome the inhibition of *sbo-alb* transcription caused by AbrB overproduction in a *spo0A* mutant background (Table 1). These results show that the Spo0-AbrB and ResDE systems of control operate independently of each other. An earlier observation that membrane-bound nitrate reductase was hyperactive in Spo0 mutants (3) was explained by possible membrane alteration in the mutant strains; however, an alternative possibility that AbrB is a positive regulator of the *narGHJI* operon is worth examining.

The results presented herein and those from previously published studies (34) provide compelling evidence that Spo0-AbrB- and ResDE-dependent mechanisms of control are exerted at the level of *sbo-alb* transcription initiation. Inspection of the *sbo-alb* regulatory region reveals a sequence extending from −37 to −62 that contains a region of approximate dyad symmetry and sequences identical to the −37 to −62 sequence within the regulatory region of the *resA* operon which is also controlled by ResDE (Fig. 2B) (2, 31). Access to the *sbo-alb* promoter region may be blocked by the AbrB protein early in the growth curve. Following Spo0A repression of *abrB* when cells approach stationary phase, ResD, activated as nitrite accumulates in the cell, will gain access to the region upstream of the promoter −35 sequence, where it will interact with RNA polymerase to stimulate transcription initiation.

Under aerobic growth conditions, Spo0A-P will accumulate as cultures approach stationary phase due to the activities of KinA, -B, -C, and -D, all of which are histidine protein kinases that donate high-energy phosphate to the Spo0 phosphorelay (6, 7, 11, 16, 17, 27, 32). The initial phosphorylation of Spo0A is thought to depend on KinC (16, 17). This results in a level of Spo0A-P sufficient for repression of the *abrB* gene. KinC has been shown to phosphorylate Spo0A in the absence of other Spo0 phosphorelay components, but its preferred target is believed to be Spo0F (11, 17). It seems that the KinC-Spo0 system is functional in anaerobically grown *B. subtilis* cells in which *sbo-alb* expression is induced. However, *B. subtilis* cells

do not readily undergo sporulation late in the growth curve when grown anaerobically in sporulation medium (data not shown; 9). It is not known if the other kinases that supply the Spo0 phosphorelay with high-energy phosphate are functional in anaerobically grown cells.

Although *sbo-alb* undergoes significant transcriptional induction when cells encounter anaerobic conditions, this is not accompanied by a proportional increase in antilisterial activity, at least not under the growth conditions used in this study (data not shown). However, an increase in the anaerobic production of subtilosin has been reported (Stein et al., 10th Int. Conf. Bacilli). Mutations in *sboA* or in any of the *alb* genes do not significantly impair anaerobic growth (data not shown). It is not obvious why the cell would benefit from the massive increase in *sbo-alb* expression during anaerobiosis. It is possible that we have not used the appropriate anaerobic growth conditions required for active subtilosin production. It is also possible that the cell accumulates inactive precursors of subtilosin, which then undergo oxygen-dependent modifications to yield an active peptide when an aerobic environment is encountered. A greater knowledge of *sbo-alb* gene products and their function, as well as an examination of other antimicrobial production systems in anaerobically grown *Bacillus* species, may provide clues as to the role bacteriocins play in the anaerobic life of bacteria.

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