JOURNAL OF BACTERIOLOGY, Dec. 1995, p. 7265–7270 Vol. 177, No. 24
0021-9193/95/$04.00+0
Copyright © 1995, American Society for Microbiology

Characterization of *Actinobacillus pleuropneumoniae* Riboflavin Biosynthesis Genes

TROY E. FULLER AND MARTHA H. MULKS*

Department of Microbiology, Michigan State University, East Lansing, Michigan 48824

Received 3 July 1995/Accepted 2 October 1995

In this paper, we report the identification, cloning, and complete nucleotide sequence of four genes from *Actinobacillus pleuropneumoniae* that are involved in riboflavin biosynthesis. The cloned genes can specify production of large amounts of riboflavin in *Escherichia coli*, can complement several defined genetic mutations in riboflavin biosynthesis in *E. coli*, and are homologous to riboflavin biosynthetic genes from *E. coli*, *Haemophilus influenzae*, and *Bacillus subtilis*. The genes have been designated *A. pleuropneumoniae ribGBAH* because of their similarity in both sequence and arrangement to the *B. subtilis ribGBAH* operon.

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia (9, 23, 39). The disease is characteristically an acute necrotizing hemorrhagic bronchopneumonia with accompanying fibrinous pleuritis (9, 39). Pleuropneumonia is an economically devastating, severe, and often fatal disease with clinical courses ranging from peracute to chronic infection (9, 15). The existence of at least 12 antigenically distinct capsular serotypes (31) has made development of a cross-protective vaccine difficult. Killed whole-cell vaccines provide, at best, serotype-specific protection (25, 26, 35, 43). In contrast, natural or experimental infection with virulent *A. pleuropneumoniae* frequently elicits protection against reinfection with any serotype (24, 25, 27). Avirulent strains of *A. pleuropneumoniae* have been tested as live vaccines and have elicited cross-protective immunity to subsequent challenge (16, 28, 44). However, the use of live vaccines in the field is problematic, particularly when the attenuating lesions in the vaccine strain have not been defined genetically. Development of attenuated strains with defined biochemical mutations that limit growth in vivo and prevent reversion to wild type is a promising approach to improved vaccines against *A. pleuropneumoniae* infection.

Riboflavin (vitamin B_2), a precursor of the coenzymes flavin adenine dinucleotide and flavin mononucleotide, is essential for basic metabolism. It is synthesized by plants and by most microorganisms but not by higher animals (1). Many pathogenic bacteria are apparently unable to utilize flavins from their environment and are entirely dependent on endogenous production of riboflavin (38). Therefore, riboflavin biosynthesis may be essential for survival of pathogens in vivo, and mutations in the riboflavin biosynthetic pathway may be attenuating.

The proposed metabolic pathway for bacterial riboflavin synthesis shown in Fig. 1 begins with GTP as the precursor (for a review, see reference 1). The most extensively studied system for bacterial riboflavin synthesis is *Bacillus subtilis* (for a review, see reference 29). The *B. subtilis* riboflavin synthesis genes are located and coregulated in an operon structure (19) that consists of five open reading frames designated *ribG*, *ribB*, *ribA*, *ribH*, and *ribT* (20, 29). The *ribGBAHT* genes encode, respectively, a *rib*-specific deaminase, the α-subunit of riboflavin synthase, a bifunctional enzyme containing GTP cyclohydrase and 3,4-dihydroxy 2-butanone 4-phosphate (3,4-DHBP) synthase activities, the β-subunit of riboflavin synthase (lumazine synthase), and a *rib*-specific reductase (29). The complete sequence of the *B. subtilis* riboflavin operon has been determined in two individual laboratories (20, 30). The *B. subtilis* structural *ribGBAHT* genes code for predicted proteins of 361 (molecular weight [MW], 39,700), 215 (MW, 23,600), 398 (MW, 43,800), 154 (MW, 16,900), and 124 (MW, 13,600) amino acids in length (20, 29). Two functional promoters have been identified in the *B. subtilis rib* operon. The main promoter, P1, for which a transcriptional start site has been determined 294 bp upstream of *ribG* (19, 30), is responsible for transcription of all five structural genes (19). Another promoter, P2, produces a secondary transcript encoding the last three genes of the operon, *ribAHT* (19). A possible third promoter that would express *ribH* has been postulated (7). In addition, the operon has been shown to be transcriptionally coregulated (30) by a *trans*-acting repressor, RibC (3, 6), which acts at a regulatory site, *ribO* (3, 21), upstream of *ribG*, apparently by a transcription termination-antitermination mechanism (29). The RibC repressor appears to respond to flavin mononucleotide and flavin adenine dinucleotide as well as to riboflavin and several of its biosynthetic intermediates and regulates expression from both *rib* P1 and *rib* P2 (4, 21, 29).

Escherichia coli is the second most chemically characterized system for riboflavin synthesis. In contrast to *B. subtilis*, the *rib* genes of *E. coli* are scattered around the chromosome and are expressed constitutively (2, 46). Rather than having a bifunctional *ribA*, *E. coli* has two separate genes, *ribB* and *ribA*, that encode the functions of 3,4-DHBP synthase (34) and GTP cyclohydrase II (33), respectively. *ribB* is homologous to the 5′ end of *B. subtilis ribA*, while *ribA* is homologous to the 3′ end (33, 34). *E. coli* genes with sequence homology to the *B. subtilis ribG* (42), *ribH* (42), and *ribB* genes have also been identified.

In this study, we have identified a fragment of *A. pleuropneumoniae* serotype 5 chromosomal DNA that triggers overproduction of riboflavin when cloned in *E. coli*. Nucleotide sequence analysis demonstrated four open reading frames with significant identity and a similar operon arrangement to that of the *ribGBAH* genes from *B. subtilis*.

Identification of a riboflavin-producing clone. A genomic library of *A. pleuropneumoniae* ISU178, a serotype 5 strain, was constructed in pUC19 and transformed into *E. coli* DH5α. A single clone, designated pTF10 (Fig. 2) and containing a 5.0-kbp insert, that produced a bright-yellow extracellular, water-

* Corresponding author. Mailing address: Department of Microbiology, Michigan State University, East Lansing, MI 48824. Phone: (517) 355-6515. Fax: (517) 353-8957. Electronic mail address: mulks@pilot.msu.edu.

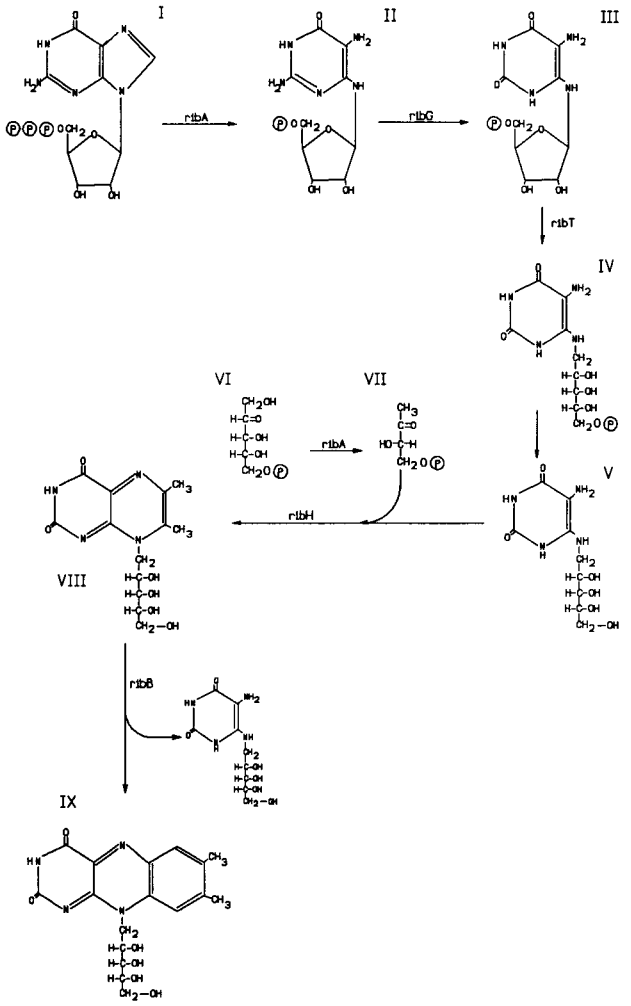

FIG. 1. Proposed bacterial riboflavin biosynthesis pathway. Proposed gene functions are as indicated, although the functions of *ribG* and *ribT* have not been determined conclusively. Structures designated with roman numerals correspond to the following: I, GTP; II, 2,5-diamino-6-(ribosylamino)-4(3H)-pyrimidinone 5′-phosphate; III, 5-amino-6-(ribosylamino)-2,4(1H,3H)-pyrimidinedione 5′-phosphate; IV, 5-amino-6-(ribitylamino)-2,4(1H,3H)-pyrimidinedione 5′-phosphate; V, 5-amino-6-(ribitylamino)-2,4(1H,3H)-pyrimidinedione; VI, ribulose 5′-phosphate; VII, 3,4-dihydroxy-2-butanone 4-phosphate; VIII, 6,7-dimethyl-8-ribityllumazine; IX, riboflavin. Structures are adapted from the work of Bacher (1).

soluble compound that fluoresced under UV light was identified. The compound was crudely purified by filtration through a 3,000-Da-cutoff membrane filter (Amicon Corp., Bedford, Mass.). Absorbance spectra of this compound in aqueous solution under neutral conditions showed peaks at 373 and 443 nm, which coalesced to a single peak at 388 nm under acidic conditions (5); these results compared well with those of a riboflavin standard. Positive- and negative-ion, fast-atom-bombardment mass spectroscopy (performed at the Michigan State University Mass Spectroscopy Facility) indicated that the compound was a flavin (data not shown). Culture of *E. coli* DH5α/pTF10 in M9 medium plus NZ amine plus 0.6% glucose yielded 10 mg of riboflavin per liter in 24 h; the A_{445} of the cell-free culture supernatant was multiplied by a factor of 31.3 to yield the riboflavin concentration in milligrams per liter (11).

Sequence of *A. pleuropneumoniae rib* genes. The nucleotide sequence of the insert in pTF10 was determined by the dideoxy chain termination method (37) with the Sequenase 2.0 kit (U.S. Biochemical, Cleveland, Ohio) and ^{35}S-dATP (Amersham Corp., Arlington Heights, Ill.), and DNA sequences were analyzed with the Genetics Computer Group (12) and GenePro (Riverside Scientific Enterprises, Seattle, Wash.) sequence analysis programs. The nucleotide sequence and corresponding predicted amino acid sequences of a 4,312-bp region of the insert in pTF10 are shown in Fig. 3. Four open reading frames of 1,232, 647, 1,205, and 461 bp that encoded proteins with predicted molecular masses of 45,438, 23,403, 44,739, and 16,042 Da, respectively, were observed. On the basis of homology with the riboflavin biosynthetic genes of *B. subtilis* (see below), these open reading frames were designated *ribG*, *ribB*, *ribA*, and *ribH*, respectively. All four open reading frames were preceded by potential ribosome binding sites, although the ribosome binding site upstream of *ribG* is not as strong as the other three. Production of riboflavin by this clone is not dependent on its orientation in pUC19 or on induction by isopropyl-β-D-thiogalactopyranoside (IPTG), indicating that it is produced under the control of a native promoter included in the cloned DNA fragment. A putative promoter sequence of the −35/−10 type (13) was identified within the sequenced region 224 bp upstream from the *ribG* start codon. This promoter, among the first described for housekeeping genes in *A. pleuropneumoniae*, is a good match for an *E. coli* consensus −35/−10 promoter. There is a 4- of 6-bp match at the −35 region, a 17-bp interval, a 4- of 6-bp match at the −10 region, an 8-bp interval, and a CAT box at the −1/+1 site. A second potential consensus promoter was identified between the genes *ribA* and *ribH*, although it is not clear whether this promoter is functional. No consensus promoter was identified between *ribB* and *ribA*, as is found in *B. subtilis*. The open reading frame encoding *ribH* is followed by an inverted repeat stem-loop structure with a ΔG of −56.0 that may function as a rho-dependent transcriptional terminator (14).

Complementation of *E. coli* mutants. The original pTF10 clone and several deletion derivatives were tested for their abilities to complement *ribA* (GTP cyclohydrase II), *ribB* (3,4-DHBP synthase), and *ribC* (α-subunit of riboflavin synthase) mutations in *E. coli* (Fig. 2). *E. coli ribA*:Tn*5* (BSV18), *ribB*:Tn*5* (BSV11), and *ribC*:Tn*5* (BSV13) mutants used for complementation studies were described by Bandrin et al. (2) and kindly provided by Barbara Bachmann (*E. coli* Genetic Stock Center, Yale University). *E. coli* strains were cultured in Luria-Bertani medium or in M9 (36) supplemented with 15 g of NZ amine (Sigma Chemical Co., St. Louis, Mo.) per liter and with riboflavin at 200 μg/ml when necessary. Complementation of the *E. coli* mutation was determined by restoration of the ability to grow on M9 minimal medium in the absence of riboflavin. Plasmids containing a complete copy of the *A. pleuropneumoniae ribB* gene complemented the *E. coli ribC* mutation. Plasmids containing the 5′ end of *A. pleuropneumoniae ribA* complemented the *E. coli ribB* mutation. Plasmids containing a complete copy of *A. pleuropneumoniae ribA* complemented both *E. coli ribB* and *ribA* mutations.

Minicell analysis. Plasmid pTF10 and its deletion derivatives were transformed into the minicell-producing *E. coli* strain DS410 (*azi-8 tonA2 minA1 minB2 rpsL135 xyl-7 mtl-2 thi-1* λ$^-$) (32), and minicells were isolated by differential centrifugation followed by glass fiber filtration as described by Christen et al. (8). The proteins encoded by these plasmids were labeled with Trans-label (^{35}S-methionine plus ^{35}S-cysteine; ICN Biomedicals, Irvine, Calif.), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12%

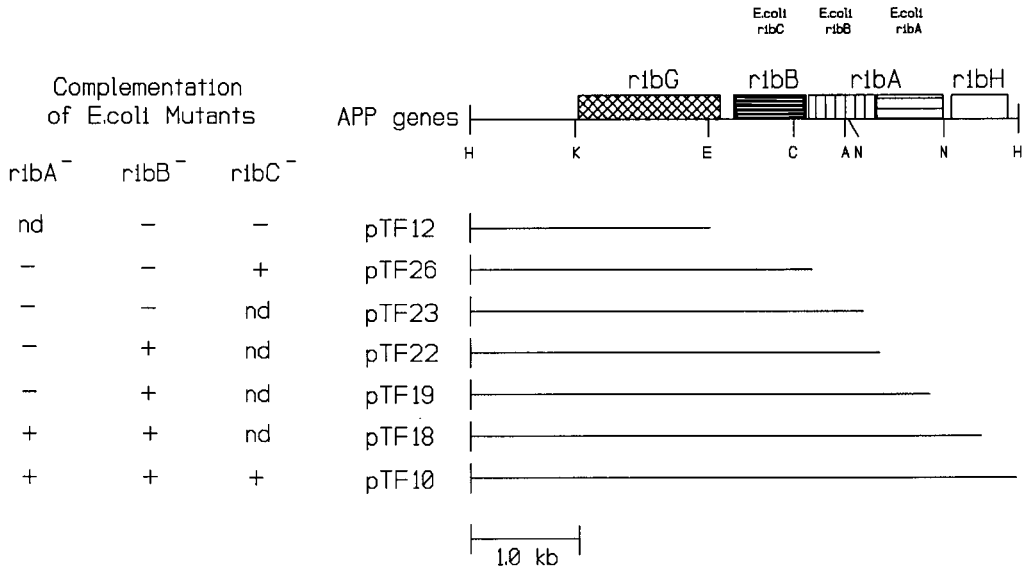

FIG. 2. Complementation of *E. coli* mutants by cloned *A. pleuropneumoniae rib* genes. A physical map for the *A. pleuropneumoniae ribGBAH* genes is shown as well as several deletions that were made from the 3′ end of the *A. pleuropneumoniae rib* clone. The *E. coli* gene designations are indicated above their *A. pleuropneumoniae* homologs. A plus indicates complementation of the indicated *E. coli* mutation by the recombinant plasmid. nd, not done. Enzyme abbreviations: A, *Ava*I; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nde*I.

polyacrylamide gel, and visualized by autoradiography. Compared with the pUC19 vector, plasmid pTF10 shows four unique proteins with apparent molecular masses of 45, 27.7, 43.7, and 14.8 kDa (Fig. 4), which correspond well with the sizes predicted for the RibG, RibB, RibA, and RibH proteins by amino acid sequence data. The RibG protein did not appear to be as strongly expressed as RibB, RibA, and RibH. Analysis of proteins encoded by plasmid pTF19 (Fig. 2), which contains no *ribH* and a slightly truncated *ribA* gene, eliminates the 14.8-kDa protein (RibH) and truncates the 43.7-kDa protein (RibA) to 42.5 kDa (Fig. 4). Plasmid pTF12 (Fig. 2), which does not contain *ribB*, *ribA*, or *ribH*, does not express the 27.7-, 43.7-, or 14.8-kDa proteins (data not shown).

Homology of *A. pleuropneumoniae rib* genes. Predicted amino acid sequences of the *A. pleuropneumoniae* RibGBAH proteins were compared with *B. subtilis* RibGBAH (20), *E. coli* RibA, RibB, RibC, RibG, and RibH (33, 34, 42), *Haemophilus influenzae* RibA, RibB, RibC, RibG, and RibE (10), *Photobacterium leiognathi* Rib I to III (17), *Photobacterium phosphoreum* Rib I to IV (18), and *Vibrio harveyi* LuxH (41) proteins with the Genetics Computer Group Gap program (Table 1). *A. pleuropneumoniae* RibG, RibB, and RibH showed distinct similarity to related enzymes from other species. *A. pleuropneumoniae* RibA showed 73% similarity to the entire RibA protein of *B. subtilis* and 61% similarity to the RibII protein of *P. leiognathi*, both of which encode a bifunctional enzyme catalyzing two distinct steps in the riboflavin pathway. In addition, the N-terminal half of *A. pleuropneumoniae* RibA, encompassing ~200 amino acids, has 59 to 65% similarity to *E. coli* RibB, *H. influenzae* RibB, and *V. harveyi* LuxH, which encode 3,4-DHBP synthase. The carboxy region of *A. pleuropneumoniae* RibA, encompassing the remaining ~200 amino acids, has 63 to 73% similarity to *E. coli* RibA, *H. influenzae* RibA, and *P. phosphoreum* RibIV, which encode GTP cyclohydrase II.

Biosynthesis of riboflavin by *A. pleuropneumoniae* appears to be more similar to that in the gram-positive bacterium *B. subtilis* than to that in the gram-negative bacterium *E. coli*. First, *A. pleuropneumoniae rib* genes are arranged in an operon similar to that seen in *B. subtilis* rather than scattered throughout the chromosome as is found in *E. coli*. However, the *B. subtilis rib* operon contains a fifth gene, *ribT*, that is proposed to mediate the third step in riboflavin biosynthesis; it is unlikely that a *ribT* homolog is present as part of the operon in *A. pleuropneumoniae* because of the presence of a strong inverted repeat following *ribH* and the lack of a likely reading frame downstream. Second, *A. pleuropneumoniae* contains a *ribA* gene that encodes a bifunctional enzyme with both GTP cy-

TABLE 1. Percent similarity of amino acid sequences of riboflavin synthesis proteins[a]

Bacterium	% Similarity with *A. pleuropneumoniae*							
	RibG		RibB		RibA		RibH	
	Compared with:	%	Compared with:	%	Compared with:	%	Compared with:	%
B. subtilis	RibG	63	RibB	69	RibA	73	RibH	83
E. coli[b]	RibG	62	RibC	58	RibB	63	RibH	74
					RibA	73		
H. influenzae[c]	RibG	58	RibC	60	RibB	65	RibE	75
					RibA	71		
P. leiognathi		NA	RibI	64	RibII	61	RibIII	69
P. phosphoreum[d]		NA	RibI	63	RibII	59	RibIII	73
					RibIV	63		
V. harveyi		NA		NA	LuxH	59		NA

[a] Identity is expressed in percent similarity as calculated by the Genetics Computer Group Needleman-Wunsch algorithm (22). Proteins with partial identity were compared with the entire appropriate *A. pleuropneumoniae* Rib protein.

[b] E. coli RibB is homologous to the 5′ end of *A. pleuropneumoniae* RibA. *E. coli* RibA is homologous to the 3′ end of *A. pleuropneumoniae* RibA.

[c] *H. influenzae* RibB is homologous to the 5′ end of *A. pleuropneumoniae* RibA. *H. influenzae* RibA is homologous to the 3′ end of *A. pleuropneumoniae* RibA.

[d] *P. phosphoreum* RibIV is homologous to the 3′ end of *A. pleuropneumoniae* RibA.

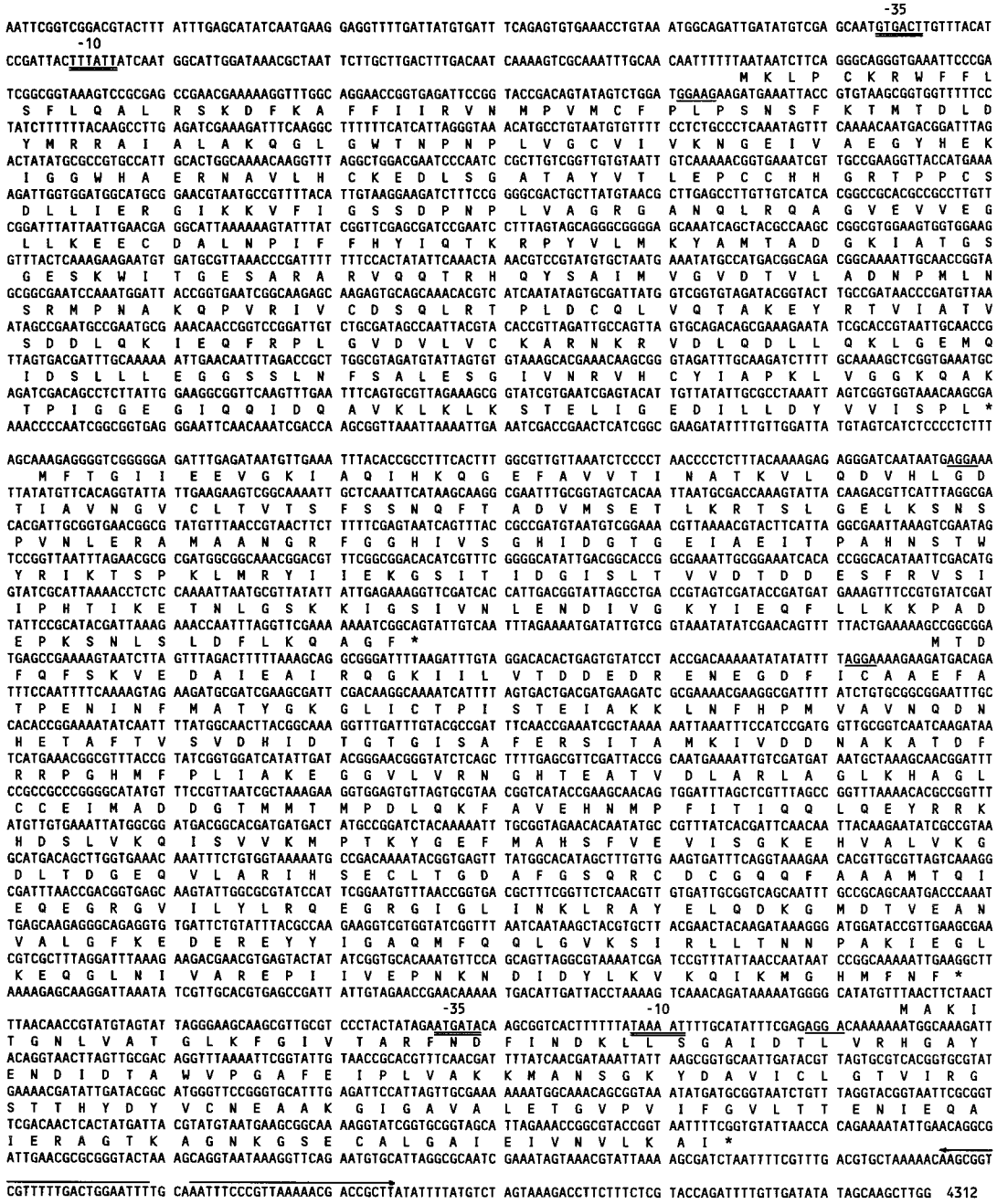

FIG. 3. Complete nucleotide sequence of *A. pleuropneumoniae ribGBAH* genes and flanking regions. The amino acid translations are shown for *ribG*, *ribB*, *ribA*, and *ribH* and correspond to bp 330 to 1560, 1685 to 2330, 2393 to 3596, and 3709 to 4168. Putative ribosome binding sites are underlined. Potential promoters for the operon and for *ribH* are double underlined. An inverted repeat that may function as a transcription terminator is indicated with arrows.

clohydrase II and 3,4-DHPB synthase activities, as is found in *B. subtilis*; *E. coli* has two genes, *ribA* and *ribB*, that separately encode these two enzymes. Finally, the *A. pleuropneumoniae* riboflavin biosynthetic enzymes are more similar at the amino acid level to the enzymes of *B. subtilis* than to those of *E. coli*, although alignment of the proteins from all three sources shows highly conserved sequences (data not shown). In contrast, riboflavin biosynthesis in *H. influenzae*, which like *A. pleuropneumoniae* is a member of the family *Pasteurellaceae*, appears to be more similar to that in *E. coli*.

It should be noted that in three bioluminescent species from the family *Vibrionaceae*, *V. harveyi*, *P. leiognathi*, and *P. phosphoreum*, riboflavin biosynthesis genes have been shown to be closely linked to the *lux* operon (17, 18, 41). Reduced riboflavin phosphate ($FMNH_2$) is the substrate for the light-emitting reaction, and therefore, an increase in bioluminescence requires an increased supply of the cofactor. Since riboflavin is the precursor for flavin mononucleotide, linkage and possibly coordinate regulation of *lux* and *rib* genes may facilitate the expression of bioluminescence in these bacteria.

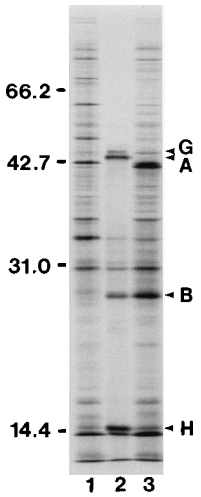

FIG. 4. Minicell analysis of pTF10 and deletions. Minicells contained pUC19 (lane 1), pTF10 (lane 2), or pTF19 (lane 3). Molecular weight standards (in thousands) are indicated on the left. Proteins encoded by the *A. pleuropneumoniae* genes are indicated by the arrows on the right. Apparent molecular masses for the *A. pleuropneumoniae* Rib enzymes are as follows: RibG, 45 kDa; RibA, 43.7 kDa; RibB, 27.7 kDa; and RibH, 14.8 kDa.

The recombinant *E. coli* DH5α containing plasmid pTF10 showed a marked increase in extracellular riboflavin production, most likely due to the lack of regulation (40) and high copy number of the cloned synthetic genes (45). Although the *A. pleuropneumoniae rib* operon is similar in structure to that of *B. subtilis*, it is not yet known whether the genes are tightly regulated in *A. pleuropneumoniae* by a repressor similar to *B. subtilis* RibC or whether they are constitutively expressed as appears to be true in *E. coli* (46). We hypothesize that *A. pleuropneumoniae* must synthesize riboflavin to meet its own metabolic demands during infection, since riboflavin is not synthesized by mammals and therefore is not likely to be freely available to *A. pleuropneumoniae* within its porcine host. Further analysis of the *A. pleuropneumoniae rib* operon should reveal interesting information on regulation and promoter and operon structure in *A. pleuropneumoniae* as well as information on the role of riboflavin biosynthesis in the pathogenesis of infections caused by this organism.

Nucleotide sequence accession number. The nucleotide sequence of the *A. pleuropneumoniae ribGBAH* genes has been submitted to GenBank and assigned the accession number of U27202.

This research was supported by a grant to M.H.M. from the State of Michigan Research Excellence Fund Center for Animal Production Enhancement. T.E.F. is the recipient of a U.S. Department of Agriculture National Needs Fellowship in Animal Biotechnology. Mass spectral data were obtained at the Michigan State University Mass Spectrometry Facility, which is supported, in part, by a grant (DRR-00480) from the Biotechnology Research Technology Program, National Center for Research Resources, National Institutes of Health.

We thank Barbara Bachmann for supplying the *E. coli* mutant strains, Vladimir Motin for interpretation of Russian papers, and Robert Hausinger, Patrick Oriel, and Douglas Gage for helpful discussion.

REFERENCES

1. **Bacher, A.** 1991. Biosynthesis of flavins, p. 215–259. *In* F. Muller (ed.), Chemistry and biochemistry of flavins, vol. 1. Chemical Rubber Co., Boca Raton, Fla.
2. **Bandrin, S. V., P. M. Rabinovich, and A. I. Stepanov.** 1983. Three linkage groups of genes involved in riboflavin biosynthesis in *Escherichia coli*. Sov. Genet. **19:**1103–1109.
3. **Bresler, S. E., E. I. Cherepenko, T. P. Chernik, V. L. Kalinin, and D. A. Perumov.** 1970. Investigation of the operon of riboflavin synthesis in *Bacillus subtilis*. I. Genetic mapping of the linkage group. Genetika **6:**116–124.
4. **Bresler, S. E., E. A. Glazunov, G. I. Chernik, T. N. Shevchenko, and D. A. Perumov.** 1973. Investigation of the operon of riboflavin synthesis in *Bacillus subtilis*. V. Flavin mononucleotide and flavin adenine dinucleotide as effectors in the operon of riboflavin biosynthesis. Genetika **9:**84–91.
5. **Bresler, S. E., V. L. Kalinin, A. S. Kriviskii, D. A. Perumov, and T. P. Chernik.** 1969. Mutant of *Bacillus subtilis* synthesizing notable amounts of riboflavin. Genetika **5:**133–138.
6. **Bresler, S. E., D. A. Perumov, G. I. Chernik, and A. P. Skvortsova.** 1976. Investigation of the operon of riboflavin synthesis in *Bacillus subtilis*. XI. Determination of the type of regulation by a test for dominance of operator-constitutive and regulator-constitutive mutations. Genetika **12:**124–130.
7. **Chikindas, M. L., G. I. Morozov, V. N. Mironov, E. V. Luk'yanov, V. V. Emel'yanov, and A. I. Stepanov.** 1988. Regulatory regions of the riboflavin operon in *Bacillus subtilis*. Dokl. Akad. Nauk SSSR **298:**997–1000.
8. **Christen, A. A., M. L. Pall, T. Manzara, and P. F. Lurquin.** 1983. Rapid isolation of *Escherichia coli* minicells by glass-fiber filtration: study of plasmid-coded polypeptides. Gene **23:**195–198.
9. **Fenwick, B.** 1994. Porcine pleuropneumonia. J. Am. Vet. Med. Assoc. **204:** 1334–1340.
10. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J.-F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenney, G. Sutton, W. FitzHugh, C. Fields, J. D. Gocayne, J. Scott, R. Shirley, L.-I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
11. **Foster, E. W., D. C. Gyure, D. L. Heefner, C. A. Weaver, M. J. Yarus, and L. A. Burdzinski.** June 1992. U.S. Patent 5,120,655.
12. **Genetics Computer Group.** 1994. Program manual for the Wisconsin package, version 8. Genetics Computer Group, Madison, Wis.
13. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. **11:**2237–2255.
14. **Holmes, W. M., T. Platt, and M. Rosenberg.** 1983. Termination of transcription in *E. coli*. Cell **32:**1029–1032.
15. **Hunneman, W. A.** 1986. Incidence, economic effects, and control of *Haemophilus pleuropneumoniae* infections in pigs. Vet. Q. **8:**83–87.
16. **Inzana, T. J., J. Todd, and H. P. Veit.** 1993. Safety, stability, and efficacy of nonencapsulated mutants of *Actinobacillus pleuropneumoniae* for use in live vaccines. Infect. Immun. **61:**1682–1686.
17. **Lee, C. Y., and E. A. Meighen.** 1992. The lux genes in *Photobacterium leiognathi* are closely linked with genes corresponding in sequence to riboflavin synthesis genes. Biochem. Biophys. Res. Commun. **186:**690–697.
18. **Lee, C. Y., D. J. O'Kane, and E. A. Meighen.** 1994. Riboflavin synthesis genes are linked with the *lux* operon of *Photobacterium phosphoreum*. J. Bacteriol. **176:**2100–2104.
19. **Mironov, V. N., M. L. Chikindas, A. S. Kraev, A. I. Stepanov, and K. G. Skryabin.** 1990. Operon organization of genes of riboflavin biosynthesis in *Bacillus subtilis*. Dokl. Akad. Nauk SSSR **312:**237–240.
20. **Mironov, V. N., A. S. Kraev, B. K. Chernov, A. V. Ul'yanov, Y. B. Golva, G. E. Pozmogova, M. L. Simonova, V. K. Gordeev, A. I. Stepanov, and K. G. Skryabin.** 1989. Genes of riboflavin biosynthesis of *Bacillus subtilis*—complete primary structure and model of organization. Dokl. Akad. Nauk SSSR **305:**482–487.
21. **Mironov, V. N., D. A. Perumov, A. S. Kraev, A. I. Stepanov, and K. G. Skryabin.** 1990. Unusual structure in the regulation region of the *Bacillus subtilis* riboflavin biosynthesis operon. Mol. Biol. Mosc. **24:**256–261.
22. **Needleman, S. B., and C. D. Wunsch.** 1970. A general method applicable to the search for similarities in the amino acid sequence of two proteins. J. Mol. Biol. **48:**443–453.
23. **Nicolet, J.** 1992. *Actinobacillus pleuropneumoniae*, p. 401–408. *In* A. D. Leman et al. (ed.), Diseases of Swine, 7th ed. Iowa State University Press, Ames.
24. **Nielsen, R.** 1979. *Haemophilus parahaemolyticus* serotypes: pathogenicity and cross immunity. Nord. Vet. Med. **31:**407–413.
25. **Nielsen, R.** 1984. *Haemophilus pleuropneumoniae* serotypes—cross protection experiments. Nord. Vet. Med. **36:**221–234.
26. **Nielsen, R.** 1976. Pleuropneumonia of swine caused by *Haemophilus pleuropneumoniae*: studies on the protection obtained by vaccination. Nord. Vet. Med. **28:**337–338.
27. **Nielsen, R.** 1974. Serological and immunological studies of pleuropneumonia of swine caused by *Haemophilus parahaemolyticus*. Acta Vet. Scand. **15:**80–89.
28. **Paltineanu, D., R. Pambucol, E. Tirziu, and I. Scobercea.** 1992. Swine infectious pleuropneumonia: aerosol vaccination with a live attenuated vaccine. Proc. Int. Pig. Vet. Soc. **12:**214.

29. **Perkins, J. B., and J. G. Pero.** 1993. Biosynthesis of riboflavin, biotin, folic acid, and cobalamin, p. 319–334. *In* A. Sonenshein (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C.
30. **Perkins, J. B., J. G. Pero, and A. Sloma.** January 1991. Riboflavin overproducing strains of bacteria. European patent application 0405370.
31. **Perry, M. B., E. Altman, J.-R. Brisson, L. M. Beynon, and J. C. Richards.** 1990. Structural characteristics of the antigenic capsular polysaccharides and lipopolysaccharides involved in the serological classification of *Actinobacillus pleuropneumoniae* strains. Serodiagn. Immunother. Infect. Dis. **4:**299–308.
32. **Reeve, J.** 1977. Bacteriophage infection of minicells: a general method for identification of in vivo bacteriophage directed polypeptide biosynthesis. Mol. Gen. Genet. **158:**73–79.
33. **Richter, G., H. Ritz, G. Katzenmeier, R. Volk, A. Kohnle, F. Lottspeich, D. Allendorf, and A. Bacher.** 1993. Biosynthesis of riboflavin: cloning, sequencing, mapping and expression of the gene encoding for GTP cyclohydrolase II in *Escherichia coli*. J. Bacteriol. **175:**4045–4051.
34. **Richter, G., R. Volk, C. Krieger, H. W. Lahm, U. Rothlisberger, and A. Bacher.** 1992. Biosynthesis of riboflavin: cloning, sequencing, and expression of the gene coding 3,4-dihydroxy-2-butanone 4-phosphate synthase of *Escherichia coli*. J. Bacteriol. **174:**4050–4056.
35. **Rosendal, S., D. S. Carpenter, W. R. Mitchell, and M. R. Wilson.** 1981. Vaccination against pleuropneumonia in pigs caused by *Haemophilus pleuropneumoniae*. Can. Vet. J. **22:**34–35.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
37. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
38. **Schott, K., J. Kellermann, F. Lottspeich, and A. Bacher.** 1990. Riboflavin synthases of *Bacillus subtilis*: purification and amino acid sequence of the α-subunit. J. Biol. Chem. **265:**4204–4209.
39. **Sebunya, T. N. K., and J. R. Saunders.** 1983. *Haemophilus pleuropneumoniae* infection in swine: a review. J. Am. Vet. Med. Assoc. **182:**1331–1337.
40. **Shavlovskii, G. M., and E. M. Logvinenko.** 1988. Flavin oversynthesis in microorganisms and its molecular mechanisms (review). Prikl. Biokhim. Mikrobiol. **24:**435–447.
41. **Swartzman, E., C. Miyamoto, A. Graham, and E. A. Meighen.** 1990. Delineation of the transcriptional boundaries of the *lux* operon of *Vibrio harveyi* demonstrates the presence of two new *lux* genes. J. Biol. Chem. **265:**3513–3517.
42. **Tetsuya, T., C. Ueguchi, K. Shiba, and K. Ito.** 1992. Insertional disruption of the *nusB* (*ssyB*) gene leads to cold-sensitive growth of *Escherichia coli* and suppression of the *secY24* mutation. Mol. Gen. Genet. **234:**429–432.
43. **Thacker, B. J., and M. H. Mulks.** 1988. Evaluation of commercial *Haemophilus pleuropneumoniae* vaccines. Proc. Int. Pig Vet. Soc. **10:**87.
44. **Utrera, V., C. Pijoan, and T. Molitor.** 1992. Evaluation of the immunity induced in pigs after infection with a low virulence strain of *A. pleuropneumoniae* serotype 1. Proc. Int. Pig Vet. Soc. **12:**213.
45. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
46. **Wilson, A. C., and A. B. Pardee.** 1962. Regulation of flavin synthesis by *Escherichia coli*. J. Gen. Microbiol. **28:**283–303.