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Cultivation of Cilia-Associated Respiratory Bacillus in Artificial Medium and Determination of the 16S rRNA Gene Sequence

TRENTON R. SCHOEB,[1]†* KEVIN DYBVIG,[1,2] MAUREEN K. DAVIDSON,[1]† AND JERRY K. DAVIS[1]†

Departments of Comparative Medicine[1] and Microbiology,[2] University of Alabama at Birmingham, Birmingham, Alabama 35294

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Cilia-associated respiratory (CAR) bacillus, an unclassified gliding bacterium associated with respiratory disease in rats, mice, and rabbits, has previously been cultivated only in embryonated chicken eggs, cell culture, or cell culture medium supplemented with conditioned medium from cultured tracheas. A reference strain of CAR bacillus, originally isolated in eggs, grew in cell culture flasks as adherent individual bacilli and ropy, whorled fascicles in cell culture media supplemented only with fetal calf serum. Using Dulbecco's minimal essential medium, we isolated CAR bacillus from naturally infected rats and a naturally infected rabbit and from experimentally inoculated mice and rats. Isolates were maintained for up to 20 passages. Isolates from rats were similar in morphology to the reference strain, but most were more actively motile and formed pincushion-like aggregates. The rabbit bacilli were smaller and formed fewer aggregates. DNAs of rat isolates differed only slightly in restriction fragment patterns from that of the reference strain, whereas that of the rabbit isolate was distinctly different. Cultures of CAR bacilli of all strains from rats contained *Mycoplasma fermentans*, *Mycoplasma pulmonis*, or both, and cultures of the CAR bacillus from the rabbit contained an unidentified arginine-utilizing mycoplasma. The sequence of the 16S rRNA gene of the reference strain was determined by amplification by polymerase chain reaction, cloning of the product, and sequencing by the dideoxynucleotide chain termination method. Comparison of the sequence with sequences in the GenBank data base indicated that CAR bacillus is a unique organism most closely related to *Flavobacterium ferrugineum* and *Flexibacter sancti*.

Gliding bacteria and their relatives are widely distributed in the environment and in the flora of humans and other animals (15). Most such organisms are not known to be pathogenic, but a few species are pathogens of fish and others may have a role in human periodontal disease or be occasional causes or complications of a variety of conditions in humans and animals (15). Cilia-associated respiratory (CAR) bacillus is an unclassified gliding and flexing filamentous bacterium associated with respiratory disease in rats, mice, rabbits, and African white-tailed rats (4, 6, 10, 11, 21, 22). It parasitizes the surface of respiratory epithelium, especially the ciliated cells; hence its colloquial designation. It is difficult to cultivate, having previously been grown only in embryonated chicken eggs, cell culture, or cell culture medium supplemented with conditioned medium from organ cultures (4, 18, 21, 22). We found that stocks of CAR bacillus derived from the original isolate (4) could be grown in various cell culture media supplemented only with fetal bovine serum and that culture in one such medium, Dulbecco's minimal essential medium (DMEM), was as efficient as culture in BALB/3T3 cell cultures for isolation of CAR bacillus from rats, mice, and rabbits. We also sequenced the 16S rRNA gene of a strain of CAR bacillus derived from the original isolate. The sequence is most closely related to those of *Flavobacterium ferrugineum* and *Flexibacter sancti*, organisms of the flavobacterium subdivision of the flavobacterium-bacteroid eubacterial phylum.

* Corresponding author.
† Present address: Division of Comparative Medicine, University of Florida, Gainesville, FL 32610.

MATERIALS AND METHODS

CAR bacillus reference cultures. Samples of CAR bacillus originally isolated in embryonated chicken eggs (4) were provided by T. Spencer, Comparative Pathology Section, National Institutes of Health. This organism was designated the reference CAR bacillus strain NIH (CARB-NIH). Another derivative of the original isolate was provided by M. LaRegina, St. Louis University, St. Louis, Mo., and was designated strain StL (CARB-StL). These were cultivated on BALB/3T3 cells obtained from, and tested for mycoplasmal contamination by, the American Type Culture Collection, Rockville, Md. The cells were grown in antibiotic-free DMEM buffered with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, Utah). The cells were further tested for mycoplasmas by fluorochrome staining (13) and by Mycoplasma TC II Rapid Detection System^r kits (Gen-Probe Inc., San Diego, Calif.) after passage in DMEM supplemented with yeast extract to enhance growth of mycoplasmas (9), by culture in glucose- and arginine-containing medium A (2) and SP-4 mycoplasma media (19), and by transmission electron microscopic examination of sediments of used cell culture medium centrifuged at 20,000 × *g*. No evidence of mycoplasma contamination of the BALB/3T3 cells was found. CAR bacilli cultured without 3T3 cells were maintained at 37°C in cell culture flasks with plug seal caps (Corning Science Products, Corning, N.Y.) and were supplied with DMEM prepared as for 3T3 cells. CAR bacillus stocks were stored at −80°C in DMEM with 20% FBS and 10% dimethyl sulfoxide. Concentrations of CAR bacillus in suspensions were

determined by counting triplicate samples in a phase hemocytometer or Petroff-Hauser counting chamber. Suspensions were sonicated at full power in a cup horn containing ice water before counting.

Culture media. All media and supplements were obtained from Sigma unless otherwise stated. DMEM with 1,000 mg of glucose per liter, DMEM with 4,500 mg of glucose per liter, Coon's modified F12, CMRL-1066, McCoy's 5A, NCTC-135, M-199, minimal essential medium with Eagle's salts, RPMI 1640, and serum-free, protein-free hybridoma medium were buffered with 25 mM HEPES. Mycoplasma media SP-4 and medium A were prepared in our laboratory. Unless otherwise stated, all media were supplemented with FBS. Solid media were prepared with SeaPlaque agarose (FMC BioProducts, Rockland, Maine).

Cultivation of CAR bacillus from animals. RAR(SD) rats and BALB/c nude mice for inoculation with CAR bacillus were obtained from the Frederick Cancer Research Facility, National Cancer Institute, Frederick, Md., and C57BL/6J obese mice were raised from breeding pairs obtained from Jackson Laboratory, Bar Harbor, Maine. RAR(SD) and C57BL/6N obese mice were used because they are reported to be susceptible to CAR bacillus (4, 6). These animals were intranasally inoculated with 10^6 to 10^7 CAR bacilli in 20 to 50 μl of culture medium while sedated with a combination of ketamine hydrochloride (Warner-Lambert Co., Morris Plains, N.J.) and xylazine (Mobay Corporation, Shawnee, Kans.), housed in microisolator cages (Lab Products Inc., Maywood, N.J.) with sterile autoclavable food, water, and bedding, and sacrificed 8 to 12 weeks after inoculation. For cultures of naturally infected animals, retired breeder albino rats of unknown strain were obtained from a conventional vendor colony known to have CAR bacillus infections. Respiratory tract lavage samples also were obtained from Sprague-Dawley rats from two conventionally maintained colonies in which CAR bacillus infection had been identified (K. R. Boschert, Washington University, St. Louis, Mo., and J. C. Murphy, Massachusetts Institute of Technology, Cambridge, Mass.). Rabbits for CAR bacillus culture had been purchased by investigators from a colony known to have CAR bacillus infection; respiratory tract lavage samples were collected when the rabbits were sacrificed according to experimental protocols. Each animal was anesthetized with pentobarbital and exsanguinated, and the trachea was aseptically exposed and clamped just below the larynx. The trachea, bronchi, and lungs were lavaged five times with nonenzymatic cell dissociation buffer (Sigma) or Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} through an intravenous catheter ligated in place. Lavage samples were centrifuged at 1,500 × *g* for 30 min, the sediments were resuspended in 0.5 ml of Hanks' balanced salt solution, and 0.10-ml portions were inoculated into 25-cm^2 flasks containing either DMEM with 10% FBS or BALB/3T3 cells supplied with DMEM. In the case of samples obtained from naturally infected rats, additional DMEM and BALB/3T3 cell cultures were supplied with medium containing 7.7 μg of trimethoprim and 38 μg of sulfamethoxazole per ml, which in preliminary experiments was found to inhibit growth of CARB-NIH only slightly. In most cases, nasal passages, larynges, tracheas, and lungs also were processed for histologic staining by the Warthin-Starry method for examination for CAR bacillus infection (21). Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Characterization of CAR bacillus strains. CARB-NIH, CARB-StL, and CAR bacillus field isolates were assessed for purity by transmission electron microscopic examination, bacterial and mycoplasma cultures, and amplification of *Mycoplasma pulmonis*-specific DNA sequences by the polymerase chain reaction (PCR). For electron microscopy, CAR bacilli were grown in DMEM and harvested by scraping and centrifuging both the bacilli and the culture medium at 20,000 × *g* for 30 min at 4°C. The resulting pellets were fixed in 1% glutaraldehyde, embedded in epoxy resin, sectioned at 80 to 100 nm, and stained with uranyl acetate and lead citrate.

Aerobic bacterial cultures of CAR bacillus samples were done in blood culture bottles, in brain heart infusion broth, on Trypticase soy agar with 5% sheep blood, and on eosin-methylene blue agar. Samples of CAR bacillus cultures of each strain also were cultured for mycoplasmas in glucose- and arginine-containing medium A, glucose- and arginine-containing medium A with fetal calf serum substituted for horse serum, and glucose- and arginine-containing SP-4 media. Cultures in each mycoplasma medium were incubated with and without 5% CO_2. Cultures showing no growth after 4 weeks were inoculated into fresh media (blind passaged). Preliminary identification of mycoplasmas isolated from CAR bacillus cultures was done by direct or indirect immunofluorescence, either of colonies on agar media (5) or of organisms grown in liquid medium, harvested by centrifugation, applied to microslides, air dried, and fixed with acetone at 4°C. Antisera were mule or donkey reference antisera from the National Institute of Allergy and Infectious Diseases, Bethesda, Md. Definitive identifications were done by immunofluorescence and growth inhibition tests conducted by J. G. Tully, Mycoplasma Section, Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Frederick, Md.

Primers for detection of *M. pulmonis* by PCR were designed on the basis of its 16S rRNA sequence and had the sequences 5′-CTGTTGCTAATACCGGATATGT-3′ and 5′-GGTACCGTCATACTTAGGG-3′. Samples of CAR bacilli for *M. pulmonis* detection were prepared by both a rapid lysis method (8) and phenol-chloroform extraction. Samples both bacilli and medium of CAR bacillus cultures were grown with or without BALB/3T3 cells and centrifuged at 20,000 × *g* for 30 min. Twenty picomoles of each primer was used per 100-μl reaction volume, with an initial incubation of 4 min at 95°C followed by 40 temperature cycles of 30 s at 95°C for denaturation, 60 s at 58°C for hybridization, and 60 s at 72°C for extension, with a final extension at 72°C for 10 min. For each sample, duplicate reaction mixtures were prepared, one of which was spiked with 0.1 pg (approximately 100 copies) of *M. pulmonis* DNA as a positive control. A 30-μl sample of each reaction mixture was subjected to electrophoresis in 2% NuSieve (FMC Bioproducts) and stained with ethidium bromide.

For comparison of restriction fragment patterns, restriction endonuclease digests of CAR bacillus DNA were prepared according to standard methods (16). CAR bacilli of each strain were grown in DMEM, harvested by scraping and centrifugation, and washed three times in STE buffer (16). DNA was isolated by chloroform-phenol extraction and treated with DNase-free RNase. Aliquots were digested with *Hin*dIII, *Eco*RV, or *Pst*I restriction endonuclease (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Samples of digests were examined by standard agarose gel electrophoresis and ethidium bromide staining.

Cloning and nucleotide sequence determination of CAR bacillus 16S rRNA gene. DNA was obtained by chloroform-

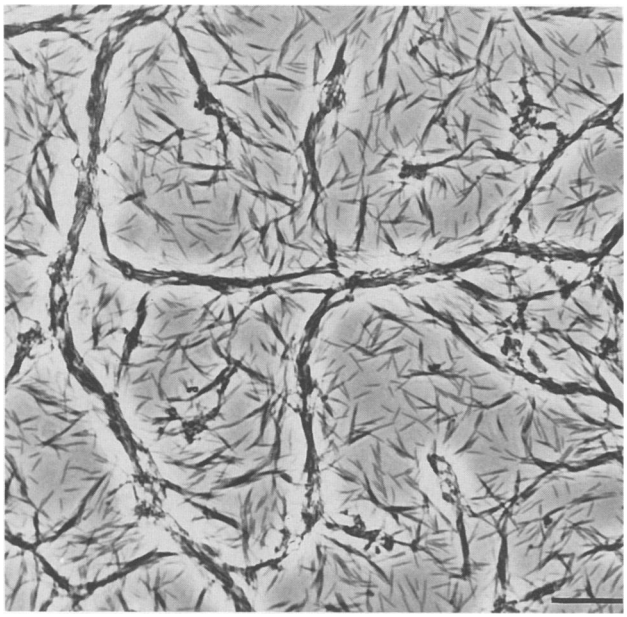

FIG. 1. Phase contrast micrograph of CAR bacillus strain NIH grown in DMEM, showing a tendency to form ropy fascicles. Bar = 18 μm.

phenol extraction from an early passage of CARB-NIH grown in DMEM without BALB/3T3 cells and washed by centrifugation three times in STE buffer. About 100 ng of DNA was enzymatically amplified by PCR under conditions recommended by the supplier of *Taq* DNA polymerase and buffer (Perkin-Elmer Cetus Corp., Norwalk, Conn.). Reaction mixtures and thermal cycling conditions were as described previously (3). PCR primers were the fD1 and rP1 oligonucleotides, which can be used to amplify nucleotides 8 through 1512 (*Escherichia coli* sequence) of the 16S rRNA gene from most eubacteria (23). A PCR product of the expected size (1.5 kb) was cloned into the *Hin*dIII-*Eco*RI site of plasmid pUC18 (primer fD1 contains an *Eco*RI site, and primer rP1 contains a *Hin*dIII site) under conditions described elsewhere (17). Plasmid DNA was maintained in *E. coli* JM103, isolated with the alkaline lysis method, and further purified by cesium chloride-ethidium bromide density gradient centrifugation (16). DNA sequencing was performed by the dideoxynucleotide chain termination method with a double-stranded DNA template and the Sequenase 2.0 kit (United States Biochemical Corp., Cleveland, Ohio). Both DNA strands were sequenced to ensure accuracy. Sequence analysis was performed with MacVector (International Biotechnologies, Inc., New Haven, Conn.) and Genetics Computer Group FASTA software.

Nucleotide sequence accession number. The sequence has been assigned GenBank accession number L11886.

RESULTS

Cultivation of CAR bacillus in artificial medium. We observed that after prolonged incubation of a culture of CARB-NIH on 3T3 cells, the 3T3 cells sloughed but many bacilli remained adherent to the flask. When supplied with fresh DMEM, the bacilli continued to proliferate, forming a confluent mat of bacilli with ropy strands of parallel bacilli in a whorling, interlacing pattern (Fig. 1). CARB-StL collected by centrifugation from medium of inoculated 3T3 cell cultures and transferred to flasks with DMEM also grew without cells; it grew in a similar pattern but somewhat more rapidly than CARB-NIH. The bacilli were easily passaged by scraping with a cell scraper and transfer to additional flasks supplied only with DMEM. In scraped flasks given fresh medium, they regrew within 5 to 10 days to densities of 10^6 to 10^7 bacilli per cm^2. Passage in this manner appeared to be repeatable indefinitely, as bacilli of both strains continued to grow well for 20 passages. Releasing bacilli from flasks by treatment with 0.25% trypsin, 0.25% proteinase K, 0.5% pronase E, and Sigma nonenzymatic cell dissociation buffer all required prolonged incubation, and bacilli so treated grew poorly.

TABLE 1. Growth of CAR bacillus in artificial media

Medium[a]	No. (log) of bacilli[a]
Coon's modified F12	9.402 ± 0.053*
CMRL-1066	8.442 ± 0.083**
M199	8.354 ± 0.062**
RPMI 1640	8.324 ± 0.072**
DMEM (4,500 mg of glucose/liter)	8.304 ± 0.178**
MEME	8.291 ± 0.132**
DMEM	8.196 ± 0.152**
McCoy's 5A	8.195 ± 0.048**
NCTC 135	8.097 ± 0.046**
Hybridoma with FBS	6.649 ± 0.526***
Hybridoma without FBS	6.508 ± 0.230***
BHI	6.376 ± 0.230***
Medium A	6.334 ± 0.357***
SP-4	6.275 ± 0.577***

[a] MEME, minimal essential medium with Eagle's salts; BHI, brain heart infusion.

[b] Means with standard deviations for three cultures 5 days after inoculation with $10^{6.3}$ CARB-NIH bacteria. Values with different numbers of asterisks are significantly different ($P \leq 0.05$).

To assess the abilities of various liquid media to support growth of CAR bacillus, 7 ml of each of the following media was added to triplicate 25-cm^2 flasks: DMEM, DMEM with added glucose, Coon's modified F12, CMRL-1066, McCoy's 5A, NCTC-135, M-199, minimal essential medium with Eagle's salts, RPMI 1640, serum-free hybridoma medium, hybridoma medium with serum added, mycoplasma medium A, SP-4, and brain heart infusion with FBS added. Except the mycoplasma media, which contained 20% FBS, and the serum-free hybridoma medium, each medium contained 10% FBS. Each flask was inoculated with 2×10^6 CARB-StL bacilli and observed daily by phase contrast microscopy at a magnification of 500×. After 5 days, growth was dense in the flasks containing F12 media, and the organisms were beginning to slough from the flasks; at this time the bacilli in each flask were harvested by scraping and counted in triplicate (Table 1). Growth was significantly better in Coon's F12 medium, but after dense growth was attained the bacilli rapidly lost viability, whereas confluent cultures could be maintained for several weeks in DMEM. Therefore, DMEM was used in subsequent experiments.

Substitution of newborn calf serum or iron-supplemented fetal calf serum for FBS or supplementation with vitamins or essential or nonessential amino acids did not improve growth of CARB-NIH or CARB-StL, nor did cultivation in vented flasks with DMEM buffered with sodium bicarbonate and incubated in 5% CO_2. Under anaerobic conditions, CARB-NIH cultured in DMEM lost viability within 5 days as indicated by detachment from the flask, loss of motility, and

TABLE 2. Isolation of CAR bacillus from animals

Host[a]	n	Infection	No. of samples positive by:	
			Histology[b]	Culture
C57BL/6N obese mice	20	CARB-NIH	0	7
RAR(SD) rats	12	CARB-NIH	12	6
BALB/c nude mice	11	CARB-NIH	8	1
Albino rats	6	Natural	6	5[c]
SD rats	3	Natural	ND	1[d]
	5	Natural	5	1[e]
NZW rabbits	16	Natural	8	1[f]

[a] SD, Sprague-Dawley; NZW, New Zealand White.
[b] Examination of tissue sections stained by the Warthin-Starry method. ND, not done.
[c] Strain X1247C.
[d] Strain X1331B.
[e] Strain X1428D.
[f] Strain X2006C.

failure to grow when cultured aerobically. Some CARB-StL bacteria incubated anaerobically remained attached after 5 days but after 10 days were almost entirely detached and immotile and failed to grow when transferred to an air environment.

To attempt to develop a solid or semisolid medium suitable for cloning, we cultured CARB-NIH and CARB-StL on DMEM and Coon's F12 media made with 0.5, 1.0, and 2.0% agarose; however, they did not grow without an overlay of liquid medium, and the organisms were diffusely distributed over the surface. We also tried cultivation of the bacilli under overlays of DMEM and Coon's F12 media containing 0.25, 0.5, and 1.0% agarose. Growth under 0.25% agarose medium was diffuse and similar to that in liquid DMEM. In medium with 0.5% agarose, there was some concentration in colony-like aggregates, but individual bacilli still were diffusely distributed throughout the culture. The bacilli did not grow in either medium with 1.0% agarose.

Cultivation of CAR bacillus from animals. Results of cultures of lavage samples from rats, mice, and rabbits are summarized in Table 2. CAR bacillus isolates from naturally infected animals were designated according to the accession numbers of the animals from which they were cultured. In one case, CAR bacillus was cultured from five of six rats; one of these, CARB-X1247C, was selected for subsequent studies. Histologic examination of respiratory tract tissues showed that in most cases, CAR bacillus was not cultured from all animals in which it was histologically evident. An exception was the group of C57BL/6N obese mice; CAR bacilli were not evident in tissues of any of these mice, although they were recovered from 7 of 20 inoculated mice.

CAR bacilli appeared in DMEM cultures within 4 days to 4 weeks. DMEM and BALB/3T3 cell cultures in which it was not found either were overgrown by bacterial contaminants or had no growth of CAR bacilli after 8 weeks, when they were discarded. In each case, bacilli in BALB/3T3 cell cultures were not evident until up to 10 days later, probably in part because they were difficult to see until they became numerous and in part because it was necessary to reduce the concentration of FBS in the culture medium for prolonged maintenance of BALB/3T3 cells. However, in all cases in which CAR bacillus was cultured in DMEM alone, it also grew in BALB/3T3 cell cultures. Bacilli in DMEM cultures first appeared clustered end-on about macrophages or cell debris adherent to the flasks and then, within a few days,

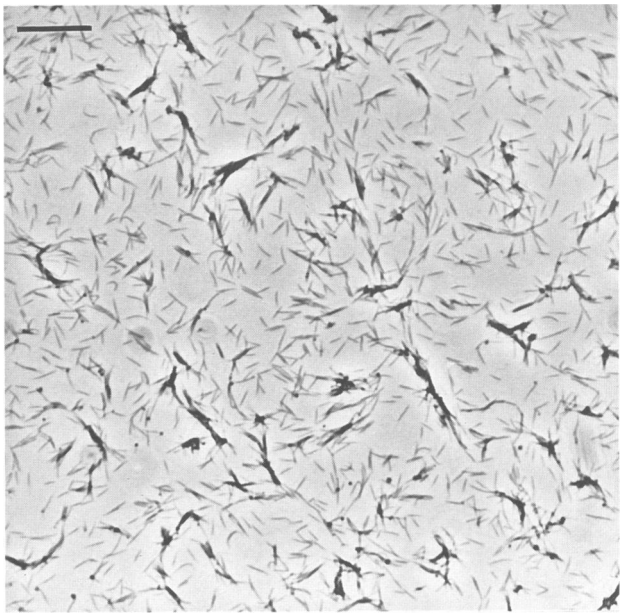

FIG. 2. Phase contrast micrograph of CAR bacillus strain X1428D grown in DMEM, showing more individual growth with a lower tendency to form fascicles than that of CARB-NIH. Bar = 18 μm.

adherent throughout the flask bottom as individual bacilli or small fascicles of bacilli (Fig. 2). Bacilli cultured from rats formed pincushion-like aggregates within 1 to 2 weeks after the organisms initially appeared, whereas those from the rabbit did not. Flexing movements and gliding motility were evident in each isolate but varied in degree. Most bacilli in CARB-X1428D and CARB-X2006C cultures flexed and glided vigorously. Bacilli in the five CARB-X1247 cultures flexed and glided less actively. Flexing and gliding bacilli were sparse in CARB-X1331B cultures. Like the reference strain, each field isolate was readily passed to additional flasks by scraping and regrew in scraped flasks to densities up to 10^7 bacilli per cm^2 within 10 days. This was repeated for 16 passages of CARB-X1247C, which by the 10th passage had assumed the ropy growth pattern typical of CARB-NIH and CARB-StL. It also became less actively flexing and gliding than in the first few passages, although it was still more motile than the reference strain, and no longer formed pincushion-like aggregates. Growth of CARB-X1331B in DMEM maintained its original pattern of sparse individual growth with a few small aggregates and poor motility for 10 passages. CARB-X1428D and CARB-X2006C maintained vigorous flexing and gliding and their original growth patterns for 11 and 12 passages, respectively.

Thawed aliquots of some frozen stocks of each strain did not grow well. Growth of these was stimulated by temporary cultivation in flasks with BALB/3T3 cells as little as 10% confluent. The bacilli could then be transferred to additional flasks without cells. The bacilli continued to grow and could be transferred without additional cells after the original cells died out upon two to four additional passages.

Characterization of CAR bacillus strains. By light and transmission electron microscopy, the morphology of the organisms of each strain was consistent with that previously described for CAR bacillus (4, 7, 10–12, 21) (Fig. 3). The organisms from the rabbit were shorter than those from rats.

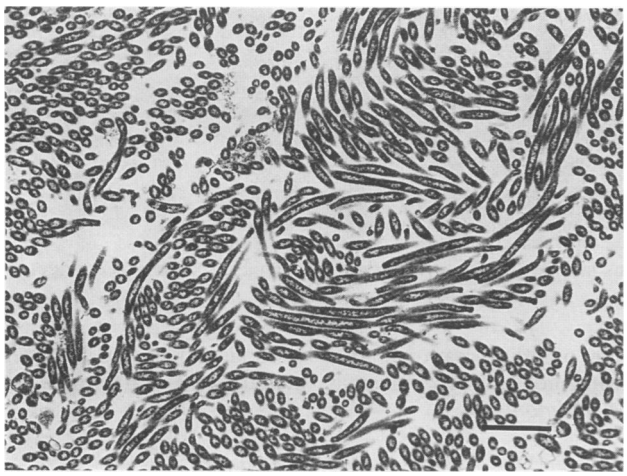

FIG. 3. Transmission electron micrograph of CAR bacillus strain NIH grown in DMEM. Bar = 1.8 μm.

In newly established cultures, the rabbit bacilli were 3 to 4 μm long, whereas those from rats were 5 to 10 μm long. With age, bacilli in all cultures increased modestly in length. After 5 to 10 days, the rabbit bacilli resembled those from rats, and occasional filaments 20 to 30 μm long appeared among the rat bacilli. DNA from all CAR bacilli from rats yielded closely similar restriction fragment patterns, whereas that from the rabbit CAR bacillus (CARB-X2006C) gave distinctly different patterns (Fig. 4).

Bacteria other than CAR bacillus were not cultured from any CAR bacillus isolate, and no organisms other than long gram-negative bacilli were evident in Gram-stained samples. However, cultures of CAR bacilli of each strain contained mycoplasmas. These were tentatively identified in transmission electron micrographs, and mycoplasmas were isolated from each CAR bacillus strain. The mycoplasma from CARB-NIH was identified as *Mycoplasma fermentans*. Mycoplasma cultures from CARB-StL contained both *M. fermentans* and small numbers (about 10^2 CFU/ml) of *M. pulmonis*. Mycoplasmas from the rat isolates CARB-X1247C, CARB-X1331B, and CARB-X1428D were identified as *M. pulmonis*. The mycoplasma from the rabbit strain of CAR bacillus CARB-X2006C grew slowly in liquid media and utilized arginine rather than glucose. We have not been able to subculture it on solid media and identify it. PCR with *M. pulmonis* primers produced products of the expected size with at least some samples of CARB-StL, CARB-X1247C, CARB-X1331B, and CARB-X1428D but not CARB-NIH or CARB-X2006C, although about 40% of the samples tested appeared to be inhibitory, with the positive control failing to yield a detectable PCR product.

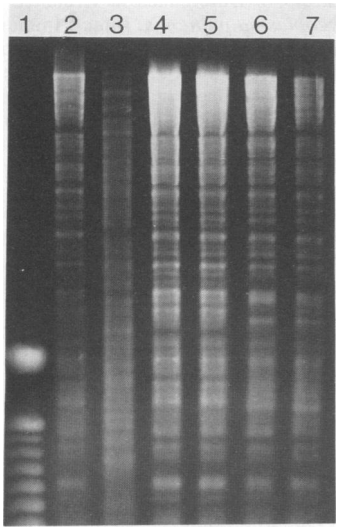

FIG. 4. Restriction fragments of CAR bacillus DNA digested with *Hin*dIII. Lane 1, 100-bp ladder; lane 2, CARB-StL; lane 3, CARB-X2006C; lane 4, CARB-X1247C; lane 5, CARB-NIH; lane 6, CARB-X1331B; lane 7, CARB-X1428D.

CAR bacillus 16S rRNA gene sequence. The sequence determined for CARB-NIH for positions 8 through 1512 is shown in Fig. 5. Comparison of this sequence with 16S rRNA sequences in the GenBank data base (release 73), using FASTA for alignment, showed it to be unique. The sequence was related to those of several species of *Flavobacterium*, *Flexibacter*, *Microscilla*, and *Cytophaga*. The greatest degrees of homology were with sequences of *Flavobacterium ferrugineum* (GenBank no. M62798) (83.5% identity among 1,495 overlapping bp) and *Flexibacter sancti* (GenBank no. M62795) (83.2% identity among 1,470 overlapping bp). MacVector analysis gave similar results.

DISCUSSION

Although growth of CAR bacillus in cell-free cultures has been reported previously (18), that medium was supplemented with conditioned medium from tracheal organ cultures. Our results show that CAR bacilli can be cultivated in a variety of ordinary cell culture media supplemented only with fetal calf serum. Furthermore, with such media, CAR bacilli were cultured from rats, mice, and rabbits, and culture in DMEM alone was as efficient as BALB/3T3 cell culture for growing CAR bacillus from animals. The apparent difficulty in culturing CAR bacillus from rabbits, with only one isolate from 16 rabbits from a colony of rabbits known to have the infection, may have stemmed from the fact that the infections were mild and of low prevalence; only eight rabbits had histologically evident infection, and in these the organisms were sparsely distributed. The organisms can be maintained in cell culture medium apparently indefinitely, inasmuch as each strain was readily maintained for 10 to 20 passages in DMEM with FBS. This simple culture system offered the advantages that prolonged maintenance of cell cultures was not necessary and the organisms were much easier to see when sparse. It also should simplify study of CAR bacillus by providing sources of bacilli free of cellular components for molecular biologic procedures and by eliminating a potential source of contamination.

According to the criteria of morphology, type of motility, and restriction fragment patterns, there was no evidence that CAR bacillus isolates obtained from naturally infected rats differed significantly from the reference strain CARB-NIH. Nonetheless, growth patterns and degree of motility varied among strains, and further analysis by Southern blotting and immunoblotting probably would reveal minor differences among isolates (24). The rabbit CAR bacillus appeared to be less closely related to the reference strain than the rat isolates, as has been observed previously (1), suggesting that CAR bacillus strains may vary among host species.

Although characteristics such as gliding motility are of little value for determining phylogeny (14, 25), the known

FIG. 5. Sequence of the positive strand of 16S rRNA of CAR bacillus strain NIH from position 8 through 1512 (*E. coli* sequence).

properties of CAR bacillus are consistent with relationships apparent from analysis of its 16S rRNA sequence. General affinities are indicated by sequence signatures of the flavobacterium-bacteroid phylum of the eubacteria (23). The CARB-NIH sequence has T, A, T, and G, respectively, at the key positions 570, 995, 1234, and 1410, a pattern typical of the bacteroid subdivision. However, CAR bacillus appears to be more closely related to organisms of the flavobacterium group than to the bacteroides. Of the 12 signature positions distinguishing the bacteroid and flavobacterium subdivisions, the CAR bacillus sequence is homologous with that of the flavobacteria at nine positions and with that of the bacteroid subdivision at only three. Computer analysis indicated affinity with several species of flavobacteria and similar organisms and with a few *Bacteroides* species. The greatest degree of homology with the CAR bacillus sequence was slightly more than 83% for sequences of *F. ferrugineum* and *F. sancti*. Thus, CAR bacillus clearly is a unique species, but a precise classification would be difficult at present, because the taxonomy of the flavobacterium group is still being developed (14, 25).

The need for extremely rigorous characterization of CAR bacillus isolates to be used for studies of pathogenicity is emphasized by the demonstration of mycoplasmal contamination of all CAR bacillus strains examined and the exhaustive testing required. Cultural confirmation was quite difficult, requiring multiple attempts and, in some cases, serial blind passages to obtain isolates of each mycoplasma that could be subcultured and identified. Furthermore, detection with PCR amplification was complicated by the large proportion of CAR bacillus samples that did not yield detectable PCR products because of inhibited amplification, including those known from culture results to contain *M. pulmonis*. Inhibition due to unknown causes is a recognized problem in amplification of mycoplasma target sequences in clinical or cell culture samples; for example, of clinical samples tested by a similar PCR method for *Mycoplasma pneumoniae*, up to one-third were found to be inhibitory (20). However, because the number of mycoplasmas in CAR bacillus cultures appeared to be very low, and because the bacilli were washed thoroughly before DNA extraction, it is very unlikely that enough mycoplasmal DNA could have been present to affect the restriction fragment patterns observed with CAR bacillus DNA. It is not possible that determination of the CAR bacillus 16S rRNA sequence was affected by presence of mycoplasmal DNA, because the 16S rRNA PCR product was cloned prior to sequencing. If mycoplasmal DNA were present in the initial PCR product and a clone containing the mycoplasmal sequence were chosen, the source of the determined sequence would be readily evident, even if the mycoplasma were an unknown species, because mycoplasmas are phylogenetically related to gram-positive bacteria. The CAR bacillus sequence clearly is related to that of other gram-negative bacteria and is very different from those of mycoplasmas, including *M. fermentans* and *M. pulmonis*.

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