Growth and Phenotypic Characterization of *Legionella* Species on Semisolid Media Made with Washed Agar

JAMES E. ROGERS,¹ GARTH W. JONES,¹ AND N. CARY ENGLEBERG^{1,2*}

Departments of Microbiology and Immunology¹ and Internal Medicine,² University of Michigan, Ann Arbor, Michigan 48109-0620

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***Legionella pneumophila* (and 28 *Legionella* species) grew efficiently on charcoal-free, buffered yeast extract medium made with washed agar and without apparent loss of infectivity for U937 cells. Because charcoal-free, buffered yeast extract is transparent, it is a suitable base for indicator media and pigment detection. In standard media, charcoal apparently prevents agar contaminants from inhibiting *Legionella* growth.**

Buffered charcoal-yeast extract agar (BCYE) (5) is used routinely for cultivation of *Legionella* species. Charcoal is used to absorb inhibitory substances generated during autoclaving (6). However, the resulting opacity of BCYE precludes its use for detection of motility, pigment, or extracellular enzymatic or hemolytic activities. We report that charcoal can be excluded if granulated agar is washed extensively in water before autoclaving.

Bacteria grown for 2 to 3 days on either BCYE or WBYE were suspended in sterile water and serially diluted to yield various inocula. Aliquots were then spread onto BCYE and WBYE plates with a glass rod. After incubation at 37°C, colonies were counted and identified as *L. pneumophila* with a serogroup 1-specific monoclonal antibody (4). In general, 21 to 24% fewer colonies of AA100 were recovered on WBYE compared with BCYE when the inoculum was grown

TABLE 1. Efficiency of plating of AA100 and AA103 on WBYE

Expt	Strain	Inoculum source	CFU counted on:		% Difference ^a
			BCYE	WBYE	
I	AA100	BCYE	105 (5) ^b	66 (4)	–21 ^c
		WBYE	116 (5)	125 (5)	+7
II	AA100	BCYE	721 (10)	547 (10)	–24
			72 (10)	57 (10)	–21
III ^d	AA103	BCYE	161 (5)	86 (5)	–47
			161 (5)	115 (5)	–29
		WBYE	184 (5)	59 (5)	–68
			184 (5)	97 (5)	–47

^a Percent difference is calculated as: $[1 - (\text{CFU}_{\text{WBYE}}/\text{CFU}_{\text{BCYE}})] \times 100$

^b Number in parentheses is the number of plates examined.

^c This value was corrected for the unequal numbers of plates counted.

^d AA103 was plated on BCYE or WBYE medium containing 300 µg of streptomycin and 20 µg of nalidixic acid per ml.

BCYE agar, with α-ketoglutarate, was prepared as reported previously (2, 5). Granulated agar, 20 g (BBL Microbiology Systems, Cockeysville, Md.), was washed five times in 2 liters of distilled H₂O and five times in deionized distilled H₂O. After drying, washed buffered yeast extract agar (WBYE) was prepared in the same manner as BCYE agar, but without charcoal.

A clinical isolate of *Legionella pneumophila* serogroup 1, AA100, and a nalidixic acid-, streptomycin-resistant derivative, AA103, passed 10 times or less on artificial media, were compared for efficiency of plating on BCYE and WBYE.

on BCYE; plating on WBYE was more efficient when the inoculum was grown on WBYE (Table 1). The relative efficiency of plating was unaffected by inoculum dilution. Plate-to-plate variation was similar for both media in experiment II (72.1 ± 6.0 on BCYE and 54.7 ± 5.6 on WBYE), suggesting that the lower recovery on WBYE was not due to occasional nonpermissive plates. *L. pneumophila* colonies grown on WBYE appeared creamy, with a birefringent edge, and grew to a size comparable to that of colonies grown on BCYE. We observed similar efficiency of plating and colonial morphology plating of *Legionella micdadei* on these media. Antibiotic-resistant strain AA103 grew with lower efficiency and at a slower rate when antibiotics were added to WBYE (Table 1, experiment III). *L. pneumophila* AA100

* Corresponding author.

FIG. 1. Zones of proteolysis or no proteolysis in legionella growing on WBYE supplemented with 1% skim milk. The two *L. pneumophila* strains tested were the protease-negative mutant PRT8 (left) and its wild-type parental strain SVIR (right).

was isolated from guinea pig splenic homogenates on both BCYE and WBYE.

To understand better the effect of washing agar, double-strength buffered yeast extract broth was either filter sterilized or autoclaved and then mixed with either washed or unwashed, autoclaved agar at 55°C. Strain AA103 grew on plates made with washed agar whether the buffered yeast extract broth was filtered sterilized or autoclaved. In contrast, AA103 failed to grow on either media made with unwashed agar, suggesting that the inhibitory factor(s) is in the unwashed agar.

To determine whether the washings contained inhibitory substances, 20 g of agar was washed with 250 ml, 500 ml, or 1 liter of deionized distilled H₂O. WBYE medium was then prepared by using each washing as the diluent. *L. pneumophila* AA100 grew as isolated colonies only on the plates made with 500-ml or 1-liter washings. We concluded that only the 250-ml wash had a sufficient concentration of inhibitor to prevent growth in reconstituted media but that the total inhibitory activity in 20 g of agar is not solubilized in a single 1-liter wash.

The inhibitory factor(s) in unwashed agar media is not freely diffusible once the media have been autoclaved. When strain AA103 was streaked across contiguous slabs (2 by 3 cm) of WBYE and buffered yeast extract (with unwashed agar), growth occurred only on the WBYE slab up to the junction of the two slabs, suggesting that the toxic factor(s) is relatively fixed.

Since cultivation of *L. pneumophila* on some charcoal-free media (e.g., supplemented Mueller-Hinton agar [8]) selects for avirulent variants, we compared WBYE- and BCYE-grown AA100 in a U937 cell model of cellular infection. Monolayers were infected at a bacterium-to-cell ratio of 10:1, and cytopathicity was quantitated in octuplicate wells after 72 h, using the vital stain MTT (3-[4,5-dimethylthiazol-

2-yl]-2,5-diphenyltetrazolium bromide) (9). BCYE- and WBYE-grown *L. pneumophila* grew similarly in cells, lysing 56.3 and 52.4% of the monolayers, respectively.

Isolates representing 28 other *Legionella* species also grew on WBYE (Table 2). Several species which had not been characterized previously for pigment production caused browning on WBYE. Two species, *Legionella birminghamensis* and *Legionella quinlivanii*, produced a distinct yellow pigment on WBYE that has not been described previously.

Secreted protease activity was demonstrated on WBYE supplemented with 1% dried skim milk (Fig. 1). An *L. pneumophila* protease mutant, PRT8 (7) (kindly provided by Paul Hoffman, Halifax, Nova Scotia), produced no clearing of the media. In contrast, its parent strain, SVIR, produced a striking zone of clearing around the bacterial growth.

These results show that *L. pneumophila* can grow on autoclaved agar media without the inclusion of absorptive components such as charcoal if toxic materials in agar are first removed by washing. Edelstein and Edelstein (3) showed a difference in the growth of *L. pneumophila* on BCYE plates made with different commercial agars. It has also been suggested that commercially available agar contains substances such as metals and other ions that inhibit the growth of some microorganisms (1). Some of these contaminants are removed with washing. For example, washing agar reduces carbon levels and permits oligotrophic bacteria to grow (unpublished data). Our data suggest that the variations in *Legionella* growth observed with different agars may be due to different degrees of contamination with inhibitory substances. However, other media might also influence the growth of *Legionella* spp. on media lacking absorbants, depending on their source and quality.

We noted that WBYE containing antibiotics was inefficient for cultivation of the antibiotic-resistant strain AA103.

TABLE 2. Pigment production by *Legionella* species on WBYE

Species tested ^a	Pigment production ^b on:	
	WBYE	BYEA ^c
<i>L. anisa</i>	+	NT
<i>L. birminghamensis</i>	Y	NT
<i>L. bozemanii</i>	—	+
<i>L. brunensis</i>	+	NT
<i>L. cherrii</i>	—	NT
<i>L. cincinnatiensis</i>	—	NT
<i>L. dumoffii</i>	—	+
<i>L. erythra</i>	+	NT
<i>L. feelii</i>	—	NT
<i>L. gormanii</i>	—	+
<i>L. hackeliae</i>	+	NT
<i>L. israelensis</i>	—	NT
<i>L. jamestowniensis</i>	—	NT
<i>L. jordanis</i>	+	+
<i>L. longbeachae^d</i>	+	+
<i>L. maceachernii</i>	—	NT
<i>L. micdadei</i>	—	—
<i>L. moravica</i>	+	NT
<i>L. oakridgensis</i>	—	+
<i>L. parisiensis</i>	+	NT
<i>L. pneumophila</i>	+	+
<i>L. quinlivanii</i>	Y	NT
<i>L. rubrilucens</i>	+	NT
<i>L. sainthelensi</i>	+	NT
<i>L. santarosensis</i>	—	NT
<i>L. spiritensis</i>	—	NT
<i>L. steigerwaltii</i>	—	NT
<i>L. tucsonensis</i>	—	NT
<i>L. wadsworthii</i>	—	—

^a Most strains were obtained from the Centers for Disease Control. The exceptions were strains of *L. oakridgensis*, *L. feelii*, *L. maceachernii*, and *L. hackeliae*, which were obtained from the Michigan Department of Public Health.

^b +, presence of brown pigment; Y, presence of yellow pigment; —, no pigment. NT, not tested.

^c BYEA, buffered yeast extract agar with supplements (from reference 10).

^d Serogroups 1 and 2.

However, the working concentrations of the antibiotics were originally determined on BCYE; thus, charcoal may adsorb some proportion of the antibiotics. Similarly constituted WBYE may contain a higher effective concentration of the drugs and be relatively inhibitory.

Since WBYE is 21 to 24% less efficient than BCYE in cultivating *L. pneumophila* grown on BCYE, we cannot

endorse WBYE for primary isolation without further modifications to improve its efficiency. However, as a transparent medium for *Legionella* cultivation, WBYE can be modified for various purposes, including more reliable antibiotic sensitivity testing, direct testing of enzymatic activities, and detection of other phenotypic traits.

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