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Importance of Carbon Dioxide in the Isolation of Pneumococci

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ABSTRACT

AUSTRIAN, ROBERT (The University of Pennsylvania School of Medicine, Philadelphia), AND PATRICIA COLLINS. Importance of carbon dioxide in the isolation of pneumococci. J. Bacteriol. **92**:1281–1284. 1966.—Of the strains of pneumococci isolated from man, 8% manifest a requirement for CO_2 if detectable growth is to occur on the surface of solid media. The pneumococci most frequently manifesting this requirement are types I, III, XVI, XXVIII, and XXXIII. These strains will grow in the presence or absence of oxygen provided the atmosphere in which they are incubated contains CO_2. Review of published data provides no unequivocal evidence for the existence of anaerobic pneumococci.

A variety of bacterial species pathogenic for man, including gonococci, meningococci, and brucellae, require a higher than atmospheric concentration of carbon dioxide for growth in or on laboratory media at the time of initial isolation. From time to time, isolated reports have appeared describing the growth of pneumococci on the surface of solid media only under anaerobic conditions (2, 9) or in an atmosphere containing a higher content of CO_2 than that found in air (10). The studies of Kempner and Schlayer on pneumococci of types I, II, and III showed that CO_2 strikingly stimulates the metabolism of these organisms in liquid media and that the observed effect is not due to alteration of the *p*H of the medium (4). Despite these scattered observations, a systematic study of the effect of various atmospheric conditions on the growth of pneumococci on solid media during primary isolation apparently has not been made, nor has an attempt been made to relate special growth requirements to specific capsular types. The present report indicates that approximately 8% of strains of pneumococci isolated from man require a concentration of CO_2 higher than that found in the normal atmosphere if identifiable growth is to occur after incubation of cultures on solid media, and that a significant proportion of certain capsular types manifest this requirement.

MATERIALS AND METHODS

Cultural techniques. Sputum collected in sterile plastic cups was emulsified with a small amount of sterile broth by refluxing in a 2-ml syringe without a needle. A loop of the emulsified sputum was streaked on the surface of each of two Trypticase Soy Agar plates (BBL) containing 5% defibrinated sheep blood. One plate was incubated in air; the other, in a closed jar in which a small candle had been allowed to burn to extinction. The plates were incubated overnight at 37 C and were examined the next day for the presence of colonies resembling pneumococci under a colony microscope at a magnification of 40 times with oblique reflected illumination. If only the plate incubated in the candle jar showed such colonies, parts of the same two pneumococcal clones were streaked on sectors of two fresh plates, each of which was incubated in one of the two atmospheres employed for primary culture. Those strains giving rise to secondary growth in pure culture only on the plate incubated in the candle jar were classified tentatively as requiring CO_2. In addition to the method just described, 1 ml of emulsified sputum was inoculated intraperitoneally into a white mouse. Mice were autopsied after death from pneumococcal bacteremia or 4 days after inoculation, and cultures of peritoneal fluid and of heart blood were incubated aerobically and in a candle jar. All strains of pneumococci were typed by the capsular precipitin or quellung reaction.

Blood from patients with pneumonia was cultured in bottles containing Trypticase Soy Broth (BBL). Cultures yielding growth were subcultured on two blood-agar plates, one of which was incubated in air and the other in a candle jar. Body fluids were streaked directly on blood-agar plates which were incubated in the same fashion.

Strains of pneumococci recovered by any of the foregoing methods were transferred to tubes of neopeptone-beef heart infusion broth containing 0.5%

defibrinated rabbit blood and were maintained at 4 C after growth.

Studies of the growth of pneumococci in a variety of atmospheres on the surface of neopeptone-beef heart infusion agar plates containing 5% defibrinated rabbit blood were performed also. Plates were incubated in air, in a candle jar, and in a Brewer anaerobic jar in which hydrogen or natural gas was combusted to remove its content of oxygen. Incubation of plates in a Brewer anaerobic jar evacuated and filled with an artificial mixture of 10% CO_2, 20% O_2, and 70% N_2 was also carried out.

RESULTS

Between 1 July 1964 and 30 June 1966, 878 strains of pneumococci were isolated from patients on the Medical Service of the Philadelphia General Hospital. Of these strains, 71 (8%) required a higher than atomospheric concentration of CO_2 for growth on the surface of solid media at the time of initial isolation. As shown in Table 1, the CO_2-requiring strains were distributed among 18 of the 82 pneumococcal capsular types, but 70% of the CO_2-requiring strains were included in capsular types I, III, XVI, XXVIII, and XXXIII. The type I strains differed from the others in that they gave rise in several instances to minute colonies approximately 1 mm in diameter after 24 hr of incubation aerobically. These clones lacked, however, the typical morphology of pneumococcal colonies and would not have been recognized as belonging to the species even when viewed through the colony microscope.

All nine strains of type XVI isolated during the period of the study showed a requirement for a higher than atmospheric concentration of CO_2 and approximately three-quarters of the strains of type XXXIII manifested a similar need. It should be noted that available typing sera did not permit distinction between capsular types XXXIII and LXVIII, and it is possible that strains classified in the former type may, in fact, represent those of two closely similar capsular types (7).

CO_2-requiring strains were virulent when sputum containing them was injected intraperitoneally into mice, and these strains could be identified in peritoneal washings by the quellung reaction. CO_2-requiring pneumococci were recovered from the blood of nine patients and grew readily in the broth inoculated with the patients' blood (Table 1). A CO_2-requiring strain of type I was isolated from the pleural exudate of a patient whose sputum and blood yielded a similar organism, and a CO_2-requiring strain of type III was recovered from the spinal fluid of a patient with meningitis. Although the strains from blood and body fluids grew adequately in liquid media, they did not produce colonies on the surface of solid media in the absence of increased CO_2 tension.

TABLE 1. *CO_2-requiring pneumococci*

Type	No. requiring CO_2	Total no. of type isolated	Per cent requiring CO_2
I	7[a]	30	23
III	12[a]	64	19
IV	2	28	—
VII	2[a]	33	—
VIII	3	42	—
IX	3	61	—
XI	1	19	—
XII	1	23	—
XVI	9[a]	9	100
XVII	4	28	—
XVIII	2	25	—
XXVIII	5	18	28
XXXIII	16[a]	22	73
XLI	1	23	—
XLIV	1	10	—
LVII	1	10	—
LXXI	1	6	—
LXXVIII	1	1	—

[a] Isolated from blood cultures as well as from sputum.

Two types of pneumococci were recovered simultaneously from the respiratory tract of several patients. From one, both pneumococci, types III and XII, were CO_2-requiring strains. In four other instances, a CO_2-requiring strain of type III, XI, or XVI was associated with a strain of type IX, XI, XIX, or XXIX which grew well on blood-agar plates incubated in air. One patient with pneumonia and bacteremia caused by pneumococcus type XVIII yielded aerobic variants from his respiratory tract but had a CO_2-requiring organism of the same capsular type in his blood.

The composition of the agar media employed and the source of blood incorporated therein did not affect the inability of the fastidious strains to grow on the surface of plates incubated in air, the requirement for CO_2 being observed when Trypticase Soy Agar or neopeptone-beef heart infusion agar was used with blood from sheep, rabbit, or man.

After one to five passages in liquid media, most of the strains requiring CO_2 on initial isolation gave rise to mutants growing normally on the surface of solid media incubated in air. Several strains of type XXXIII, however, failed to manifest this phenomenon.

To determine more precisely the nature of the gaseous environment required for the growth of pneumococci recovered from plates incubated in a candle jar, organisms on the surface of neo-

TABLE 2. *Surface growth of pneumococcal variants in different atmospheres*

Atmosphere	Growth		
	CO_2-requiring pneumococci	Aerobic mutant of CO_2-requiring pneumococci	Aerobic pneumococci
Air	−	+	+
Candle jar	+	+	+
10% CO_2, 20% O_2, 70% N_2	+	+	+
Anaerobic jar, H_2	−	+	+
Anaerobic jar, natural gas	+	+	+

peptone-beef heart infusion blood-agar plates were incubated in five different atmospheres (Table 2). The pneumococci grew well in a candle jar, in a normal atmospheric concentration of oxygen in which part of the nitrogen had been replaced by CO_2, and in an anaerobic jar to which natural gas had been added yielding CO_2 on combustion. When hydrogen was substituted for natural gas to yield water instead of CO_2 in removing oxygen from the atmosphere, the pneumococci failed to grow just as they did in air. In contrast to these findings, pneumococci growing aerobically on initial isolation and aerobic mutants of the CO_2-requiring strains grew well on plates incubated in air or in an anaerobic environment developed with hydrogen and lacking CO_2.

DISCUSSION

A number of earlier reports have described strains of pneumococci unable to grow on the surface of solid media in air (2, 9, 10). These strains have been said to require either anaerobic conditions or an increased tension of atmospheric CO_2 for growth. To cite several examples, Smith described two strains of pneumococcus which grew initially only under the anaerobic conditions in a McIntosh and Fildes jar but gave rise subsequently to variants which would grow in air. Interestingly, one of these two strains cross-reacted in agglutination tests with pneumococcus type XVI (9). Carlens, in a study of otitis media caused by pneumococcus type III, noted that anaerobic conditions were of great importance in the isolation of this organism, "for, in several cases, the type 3 pneumococcus grew only on the anaerobic plate" (2). The means of achieving anaerobiosis is not stated in his report. Walter et al. (4), in their initial description of pneumococcus type XXXIII, made the following observation: "Freshly isolated strains of type 33, when plated on solid media, often grow better when incubated in an air-tight container in which a wax candle is burned until extinction, than under atmospheric conditions." Rockwell, in his studies on the influence of carbon dioxide on the growth of bacteria, observed that a strain of pneumococcus isolated from joint fluid would grow anaerobically in the presence of carbon dioxide but not in its absence or in air (8). From these and other published observations, it would appear that there is little, if any, evidence for the existence of anaerobic pneumococci. Where methods for achieving anaerobic conditions for the growth of pneumococcus have been described, it is clear either that CO_2 was present and necessary for growth or that the method for eliminating oxygen resulted probably in the simultaneous generation of CO_2.

The results of the present study suggest that oxygen is not inimical to the growth of pneumococci when catalase is present, but that some strains require a concentration of CO_2 in excess of that present in air. Although the minimal and optimal concentrations of CO_2 for the growth of pneumococci on solid media have not been determined, the studies of the metabolism of pneumococci in liquid media by Kempner and Schlayer (4) showed marked stimulation over concentrations ranging from 1 to 20%, with an optimal effect at 10%. The appearance of pneumococcal colonies on plates incubated in candle jars and in jars in which anaerobic conditions were developed by combustion of natural gas did not differ significantly from those on plates incubated aerobically in an atmosphere of 10% CO_2. Both in liquid (4) and on solid media, the lowering of the *p*H of the media by CO_2 is more than offset by the growth-promoting effect of the CO_2.

The metabolic pathway into which CO_2 enters to facilitate the growth of pneumococcus, like that in meningococcus and brucella, has not been defined. Addition of sodium bicarbonate to the medium does not replace the requirement for gaseous CO_2. Studies of a variety of streptococcal strains of Lancefield's groups B, F, and G have shown that some have a similar requirement for CO_2 (1, 3, 5), and experiments with isotopically labeled CO_2 have suggested that a one-carbon compound condenses with a three-carbon compound to form oxaloacetic acid which is then aminated to form aspartic acid, with most of the label being found in this amino acid (6, 11).

If pneumococcus is to be cultured from the sputum and body fluids of man only on the surface of solid media, it is essential, if all strains are to be recovered, that such media be incubated

in an atmosphere, aerobic or anaerobic, containing CO_2. Aerobic incubation alone or incubation under anaerobic conditions generated with hydrogen will result in failure to recover 5 to 8% of the pneumococcal strains present in such specimens. Pneumococci requiring CO_2 can be isolated, however, from cultures in liquid media incubated aerobically or from the body fluids of mice injected intraperitoneally with specimens containing such strains.

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