Growth of Cell Wall-Defective Variants of *Escherichia coli*: Comparison of Aerobic and Anaerobic Induction Frequencies

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A method for quantitating the conversion of *Escherichia coli* to colony-forming, cell wall-defective (CWD) bacteria has been developed. The induction frequency, i.e., the percentage of the population recovered as CWD colonies was determined for 20 randomly selected clinical isolates of *E. coli* under aerobic and anaerobic incubation conditions. Penicillin (1,000 U/ml) was the inducing agent. The 20 strains segregated into three groups. Group I organisms produced CWD colonies with high frequency both aerobically and anaerobically. Group II organisms showed a much higher induction frequency anaerobically than aerobically. Group III organisms were poor inducers. Thirty percent of the strains were group I, 50% were group II, and 20% were group III organisms. These data indicate that anaerobic conditions enhance the induction and growth of CWD *E. coli* in the research laboratory and suggest that anaerobic incubation may be important in recovery of medically significant CWD bacteria.

The existence of cell wall-defective (CWD) bacteria has long been thought to be a plausible explanation for persistence of bacteria after an apparently appropriate antibiotic treatment regimen. An assessment of their importance in clinical medicine awaits the development of optimal media and incubation conditions for CWD bacteria.

Quantitation of CWD bacteria in clinical specimens is rarely done, nor has a quantitative evaluation of atmospheric conditions in the induction or recovery of CWD organisms been reported. Early reports by Dienes, Weinberger, and Madoff (4, 15) state that anaerobic conditions are necessary for induction and growth of L-forms of *Salmonella* and *Shigella*. Dienes and Sharp (3) found that the requirement for anaerobiosis for induction of hemolytic streptococci was related to medium batch variation. Freimer et al. (6) reported that anaerobic incubation was necessary for induction but not subculture of the L-phase of streptococci. Landman and Ginoza (10) showed no effect of lowered oxygen tension on the growth of L-forms of *S. paratyphi*. Landman et al. (9) obtained a quantitative conversion of *Proteus mirabilis* and *Escherichia coli* to L-forms with aerobic incubation. Nimmo and Blazevic (13) reported that the growth rate of previously induced L-forms of *E. coli* was greater with aerobic incubation. Lederberg and St. Clair (11) reported strain variation in the ability of *E. coli* to grow as L-

form colonies but found no special atmosphere necessary for L-type growth.

With the exception of a report by Seeburg (14), the current consensus seems to be that aerobic conditions are optimal for induction and growth of CWD forms of bacteria. Seeburg (14) reported that anaerobic conditions were superior for the induction of most strains of *E. coli*, but he did not report quantitation of induction or recovery.

We chose to evaluate media and conditions suitable for the induction and growth of CWD *E. coli*. *E. coli* was chosen because it is the most frequent cause of urinary tract infections, a site in which many investigators have suggested that CWD bacteria could be important. Initial attempts in our laboratory to quantitate the ability of a random isolate of *E. coli* to produce CWD colonies, using aerobic incubation, were unsuccessful for lack of countable plates. CWD growth developed only with large inocula, and only subsurface growth occurred. Anaerobic incubation of dilution pour plates resulted in greater yields of CWD colonies and relieved the large inoculum requirement. These findings led us to a quantitative study of the effects of anaerobic incubation on the yield of penicillin-induced, CWD *E. coli*.

MATERIALS AND METHODS

Organisms. Sources of the 20 random clinical isolates of *E. coli* were urine (G598, G584, G552, G745,

G906, G956, P629, E970, E1024, E1045, and E1065), feces (P646, P624, P765, and P790), exudates (E740 and E964), genital swab (G647), and others (P537 and P538). Antibiotic susceptibility tests were by the procedure of Bauer and Kirby (2) and showed that all the strains except G906 were sensitive to ampicillin. All cultures were biochemically typical of *E. coli* by appearance on MacConkey, triple sugar iron, and lysine iron agars, by tests for citrate utilization, and by urease and tryptophanase production (5).

Inoculum cultures. Cells to be used for induction studies were grown in 50 ml of nutrient broth (Difco) in a 250-ml Erlenmeyer flask. Cultures were incubated stationary at 35°C for 24 h.

Media. Normal plate count medium consisted of brain heart infusion broth (BHI) with 1.5% agar. Induction medium for the support of CWD cells was BHI, 3.7%, agar, 1.0%; sucrose, 10%; MgSO_4 , 0.125%; and penicillin G, 1,000 U/ml. The sucrose- MgSO_4 solution was prepared double strength, autoclaved separately, and added aseptically to double-strength BHI agar at 50°C. Penicillin G was added to a final concentration of 1,000 U/ml just prior to pouring induction plates. BHI-penicillin control plates consisted of BHI, 3.7%, agar 1.0%; and penicillin, 1,000 U/ml.

Demonstration of cell wall defectiveness. Surface colonies on induction plates were observed for typical "fried egg" L-type colonies. Subsurface CWD colonies were distinguished from normal colonies by their diffuse edges as well as their spherical shape. Colonies were verified as consisting of CWD cells by observation of agar-squash preparations with phase-contrast microscopy.

Determination of incubation period. Six sets of induction plates were inoculated with appropriate dilutions of *E. coli* P629 and P538. Triplicate plates were incubated anaerobically (GasPak anaerobe jars; Baltimore Biological Laboratories), for 3, 4, 5, 6, 7, and 10 days. Yields of CWD colonies were determined by counting colonies with the aid of a Lab-Line 1585 colony counter.

Determination of induction frequency. Tenfold serial dilutions of the 20 inoculum cultures were prepared in nutrient broth dilution blanks. Triplicate pour plates of 10^{-1} to 10^{-7} dilutions were prepared with normal plate count medium or induction medium. BHI-penicillin-agar pour plates were inoculated with 1 ml of the 10^{-1} dilutions to demonstrate penicillin sensitivity. Sets of induction plates were incubated aerobically and anaerobically. Plates for normal bacterial counts were incubated aerobically. Colony counts were made after 3 days of incubation at 35°C. Induction frequency was defined by the formula:

$$\text{Induction frequency} = \frac{\text{average CWD colony count}}{\text{average normal colony count}} \times 100$$

Effects of aerobic exposure prior to anaerobic incubation. Seven triplicate sets of induction plates inoculated with dilutions of *E. coli* P629 were exposed to aerobic conditions prior to being placed in an anaerobe jar. One set was placed in the anaerobe

jar as soon as the agar solidified, and another set was incubated entirely aerobically. The remaining sets were incubated aerobically for 5, 8, 12, 24, or 48 h prior to anaerobic incubation. All colonies were counted at 96 h.

Effect of length of anaerobic incubation. Six triplicate sets of induction plates inoculated with dilutions of *E. coli* P629 were incubated anaerobically for intervals of 5, 8, 12, 24, 48, and 96 h before being incubated aerobically. Colonies were counted at 96 h.

RESULTS

Demonstration of cell wall defectiveness.

Figure 1a shows a typical L-form colony that resulted from surface growth on induction plates. Figure 1b shows the morphology of subsurface CWD colonies. Figure 2 depicts typical CWD cells in agar-squash preparations as seen by phase-contrast microscopy. In no case did mixtures of CWD and bacilliform colonies occur concomitantly.

Determination of incubation period. Table 1 shows the effect of extended incubation on yields of *E. coli* P629 and P538 CWD colonies. Neither the relatively high yields from P629 nor the relatively low yields from P538 were increased by incubation longer than 3 days.

Determination of induction frequency for 20 isolates of *E. coli*. Induction frequencies resulting from both aerobic and anaerobic incubation for clinical isolates of *E. coli* are shown in Table 2. The induction frequencies segregate the organisms into three distinct groups: (i) group I, relatively high inducers aerobically and anaerobically; (ii) group II, high anaerobic, low aerobic inducers; and (iii) group III, low inducers regardless of atmospheric conditions. Of the 20 isolates tested, 30, 50, and approximately 20% exhibited group I, II, and III characteristics, respectively. One isolate, G906, which was resistant to ampicillin, did not become CWD and, as would be expected, grew on BHI-penicillin sensitivity control plates. Anaerobic incubation enhanced the growth of CWD organisms of all groups as judged by increased colony size. Group II organisms produced up to 86,000 times more CWD colonies anaerobically. This tremendously increased yield suggested the following experiments to define requirements for anaerobic incubation in group II organisms.

Effect of aerobic preincubation on anaerobic induction frequency. The effect of aerobic incubation prior to anaerobic incubation is shown in Fig. 3. The induction frequency of *E. coli* P629 decreases linearly over the first 12 h of aerobic exposure. After 12 h, the yield of CWD colonies tapers to that obtained with total aro-

bic incubation. These results indicate that maximal induction of group II organisms depends upon prompt establishment of anaerobic conditions.

Effect of duration of anaerobic incubation on induction frequency. Table 3 shows that 48 h of anaerobic incubation are required for the appearance of optimal yields of macroscopically countable CWD colonies of *E. coli* P629. The reduced yields with less than 24 h of incubation and the observation that colonial growth ceased

with aerobic conditions suggest that group II organisms require anaerobic conditions to grow in the CWD state.

DISCUSSION

The ability of an organism to produce CWD colonies may be influenced by whether it is a recent isolate or an old laboratory strain (8). Dienes et al. (4) reported that six freshly isolated strains of *S. typhi* produced abundant L-type colonies, whereas an old laboratory strain

FIG. 1. Morphology of CWD cells photographed at $\times 40$. (a) Typical L-form colony of surface growth; (b) subsurface CWD colony.

FIG. 2. Typical CWD cells in agar-squash preparations as seen by phase-contrast microscopy ($\times 1,000$).

produced L-type colonies poorly. For this reason, *E. coli* organisms freshly isolated from clinical specimens were chosen for study. Furthermore, fresh isolates should more closely approximate the inducibility of organisms in vivo than old laboratory strains.

Misuse of the terms "protoplast, spheroplast, transitional forms, and L-forms" can lead to confusion concerning osmotically fragile forms of bacteria unless terms are carefully defined. We have used the term CWD variant or form for our penicillin-induced, osmotically sensitive *E. coli* in accordance with the definition by McGee and Wittler (12). These authors define CWD variants as forms of organisms that exhibit changes consistent with damaged or defective cell walls with no implication of a change in genetic constitution. Furthermore, they describe "L-phase variants or L-forms" as a specific type of CWD variant that exhibits a typical "fried egg" colonial morphology. "Unstable L-phase variants" are those variants that revert to bacterial phase upon removal of the inducing substance.

In the classical sense, the CWD forms produced by *E. coli* under our test conditions would best fit the description of unstable L-phase variants. Osmotic fragility was demonstrated by lack of growth in nonsucrose-containing controls as well as typical macro- and microscopic

appearance of the osmotically protected colonies. Phase-contrast microscopy of CWD colonies after prolonged (1 week or more) incubation in induction medium revealed spherical cells that had developed bacillary "tails" resembling forms described as reverting L-forms by Lederberg and St. Clair (11). Transfer of these CWD colonies to hypotonic medium resulted in normal bacilliform colonies. Removal or inactivation of the inducing agent is a prerequisite for reversion. Destruction of penicillin with prolonged incubation was probably affected by penicillinase, which Ayliffe (1) found to be present in low levels in all of the 41 strains of *E. coli* that he tested.

The mechanism of anaerobic enhancement of growth and yields of CWD *E. coli* can only be

TABLE 1. Determination of incubation period required for maximal yields of CWD *E. coli*

Incubation time (days)	Yield of CWD colonies with <i>E. coli</i> :	
	P629 $\times 10^8$	P538 $\times 10^4$
3	3.8	5.4
4	3.9	7.0
5	4.0	4.6
6	3.1	4.7
7	3.6	8.4
10	4.1	7.1

TABLE 2. Aerobic versus anaerobic incubation for induction and growth of CWD variants of *E. coli*

Group	Organisms	Induction frequency (%)	
		Aerobic	Anaerobic
I	G598	10	24
	P646	8	12
	P624	37	39
	E970	4	5
	P765	2	10
	P790	7	16
II	G647	0.001	6
	G584	0.0003	26
	P537	0.0006	18
	G552	0.0006	21
	P629	0.002	30
	E740	0.002	1
	E964	0.002	1
	E1024	0.0005	11
	E1045	0.0003	0.4
	G956	0.001	15
III	P538	0.0005	0.001
	G745	0.0001	0.001
	E1065	0.00004	0.003

FIG. 3. Effect of aerobic preincubation on the anaerobic induction frequency of *E. coli* P629.TABLE 3. Duration of anaerobic incubation required for optimal growth of CWD *E. coli* P629

Time (h)	Avg plate count at 10 ⁻⁶
0	0
5	0
12	0
24	Too small to count
48	70 ^a
96	66

^a Colonies smaller than at 96 h of anaerobic incubation.

speculated upon. Aerobic conditions inhibit the growth of CWD group II organisms. Gregory, Yost, and Fridovich (7) demonstrated lethal effects of hyperbaric O₂ to normal *E. coli* cells. In addition, they described two superoxide dismutases that protect the cells from O₂⁻, the probable cause of O₂ toxicity. One of the enzymes

containing iron as the prosthetic metal is located in the periplasmic spaces of *E. coli*. Conversion of the cells to CWD forms might result in dispersion of the periplasmic superoxide dismutase, rendering them more susceptible to the toxic effects of oxygen. At any rate, anaerobic incubation has a profound effect on the induction and growth of CWD *E. coli*. Eighty percent of the strains produced high yields of CWD colonies under anaerobic incubation. The formation of CWD colonies was observed in all 19 ampicillin-sensitive strains tested. Half of the strains (group II) required anaerobiasis for abundant yields of CWD colonies, whereas only 30% of the strains fared as well aerobically. In no case did aerobic incubation increase the induction frequency. These findings conflict with the reports that anaerobic conditions do not benefit the growth of CWD bacteria (9, 10, 11, 13). This disagreement probably stems from other investigators selecting species or strains with a propensity for producing aerobic CWD forms or fortuitously choosing an organism with the qualities of our group I organisms. A clinical trial using anaerobic conditions for the isolation of CWD *E. coli* is indicated as well as further investigation into the effects of anaerobiasis on other organisms.

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