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Ecological Relationships of Bacteria Involved in a Simple, Mixed Anaerobic Infection

D. MAYRAND[1]* AND B. C. McBRIDE[2]

Département de Biochimie (Sciences), Université Laval, Québec G1K 7P4,[1] and Department of Microbiology, University of British Columbia, Vancouver V6T 1W5,[2] Canada

Infectivity of *Bacteroides asaccharolyticus* (formerly *B. melaninogenicus* subsp. *asaccharolyticus*; see S. M. Finegold and E. M. Barnes, Int. J. Syst. Bacteriol. **27**:388-391, 1977) was dependent on the presence of a second organism. An infective consortium consisting of *B. asaccharolyticus* and *Klebsiella pneumoniae* was defined. Neither organism was infective alone, but the *Klebsiella* could be replaced by organisms of a number of different genera. The nature of the infection appeared to be determined by the length of the lag period preceding the initiation of growth of *B. asaccharolyticus*. A rapid onset of growth led to the severe spreading form of the disease, whereas a slow initiation of growth resulted in the formation of a localized, self-limiting abscess. *B. asaccharolyticus* depends on the second or "helper" organism to produce a required growth factor which is not present at the inoculation site. The growth factor was shown to be succinate which was able to replace the hemin requirement. The dependency on succinate produced by *K. pneumoniae* was demonstrated in agar medium, in liquid culture, and in the infectivity assay. Any organism which produced succinate was able to stimulate growth of *B. asaccharolyticus* on agar medium and could replace *K. pneumoniae* as a member of the infectious consortium. The need for the second organism could be eliminated by inoculating *B. asaccharolyticus* together with agar-immobilized succinate or hemin.

Anaerobic bacteria comprise a large percentage of the gingival microflora, and it is not unreasonable to assume that they may be involved in the initiation of pathogenic processes. Often, infections involve several species or genera of bacteria that behave in a cooperative fashion to produce sepsis. Examples of such infections include peritonitis (1) and gingivitis (11). Cooperative or synergistic infections have been studied in a number of animal model systems (7, 17) and in a natural infection, sheep foot rot (19).

Successful transmissible infections in animals inoculated with defined mixtures of microorganisms were reported by several investigators (8, 11, 15, 21; R. C. Kestenbaum and S. Weiss, Int. Assoc. Dent. Res., abstr. no. 14, 1962). The typical mixed anaerobic infection using gingival crevice material was first described by MacDonald et al. (12, 13). The infection could be of two types: (i) a localized abscess containing exudate material which could be used to transmit the infection to a second animal, and (ii) a rapidly spreading necrotic infection which perforated either the skin or the abdominal wall. In the latter case the animal died almost immediately. Subsequent studies (11, 21) indicated that *B. melaninogenicus* played an essential role in the infection, whereas the role of some of the other organisms has as yet only been partially defined (4, 21-23, 25). The aim of this study was to gain an understanding of the ecological relationships which exist among the bacteria required to initiate and sustain a defined, mixed anaerobic infection involving *B. asaccharolyticus*.

MATERIALS AND METHODS

Bacterial culture. *B. asaccharolyticus* strains K110 and CR2A were obtained from P. A. Mashimo. These are collagenolytic strains originally isolated by MacDonald and co-workers (12) from patients with diagnosed gingivitis. Other strains of *B. asaccharolyticus* were isolated in our laboratory from human and dog gingival scrapings. Samples were streaked on freshly poured blood agar plates. After 4 to 7 days of anaerobic incubation at 37°C, black colonies were picked from the plates and subcultured on the same medium until pure cultures were obtained. Isolation of vitamin K-requiring strains of *B. asaccharolyticus* was performed as follows: the *B. asaccharolyticus* were streaked on blood agar plates and the plates were then inoculated with a central streak of *Staphylococcus aureus*. Growth of vitamin K-requiring strains was evident near the *S. aureus* streak.

Five different isolates numbered 20, 21, 22, 23, and 25 were isolated from high-dilution plates of exudate material. Colonies were subcultured aerobically and

anaerobically and originally characterized by their Gram reaction and cellular morphology. Isolate 20 was further characterized by standard procedures (18) and proved to be *Klebsiella pneumoniae*. *Fusobacterium nucleatum*, *Veillonella alcalescens*, and *Streptococcus sanguis* were isolated from human dental plaque and identified by standard morphological, nutritional, and metabolic end product analysis. *Actinomyces viscosus* 15987 was obtained from the American Type Culture Collection. *Escherichia coli* was obtained from the departmental collection. *Staphylococcus albus* was isolated from mannitol salt plates inoculated with skin swabs. Strain OBM-3 was isolated from the human gingival crevice and characterized as a saccharolytic strain of *B. melaninogenicus*.

Cultural methods. Tubes or plates were incubated in an anaerobic glove box (Coy Manufacturing, Ann Arbor, Mich.) containing an atmosphere of $N_2/H_2/CO_2$ (85:10:5) at 37°C.

Liquid cultures of *B. asaccharolyticus* were maintained in the hemin-Trypticase-yeast extract medium described by Gibbons and MacDonald (5). A basal medium (pH 7.0) consisting of Trypticase (17 g/liter), yeast extract (3 g/liter), K_2HPO_4 (2.5 g/liter), and NaCl (5 g/liter) was utilized to deplete *B. asaccharolyticus* of its endogenous supplies of hemin. Cells obtained from the basal medium cannot grow when subcultured a second time in hemin-free medium.

For some experiments, the following supplements were added separately or together to the basal medium: vitamin K_1 (Sigma Chemical Co., St. Louis, Mo.) 10^{-3} g/liter; glucose (Fisher Scientific Co.) at 1 g/liter; hemin (Sigma) 5×10^{-3} g/liter; succinic acid (Eastman Organic Chemicals, Rochester, N.Y.) (see below for concentrations). In all cases, the compounds were added to the medium and the pH was adjusted to 7.0 before sterilization.

K. pneumoniae was grown in Trypticase soy broth or in the glucose-supplemented basal medium described above. All other organisms were maintained on glucose- or brain heart infusion-supplemented basal medium.

Infectivity assay. Cells to be used for inoculation of animals were harvested from blood agar plates or from broth cultures and suspended in 1 ml of sterile phosphate-buffered saline containing 0.05% sodium thioglycolate, pH 7.0. Guinea pigs weighing 150 to 200 g were injected subcutaneously in the groin area with 0.5 ml of either in vitro-cultured cells or exudate aspirated from an infected guinea pig and observed for up to 4 weeks. The criteria for evaluating a positive infection were: (i) the presence of an abscess (pustular or necrotic) and (ii) the transmissibility of the disease. The latter was demonstrated by injecting material aspirated from a lesion into a second animal to produce a similar pathology. This process was repeated at least twice to establish that the cells were growing. Exudate was aspirated from infected guinea pigs by using a sterile disposable syringe while the animal was under light ether anaesthesia. The exudate was examined for microbial contamination by plating on blood agar and incubating it aerobically and anaerobically. The criteria used to characterize an infection are as follows: (−), no evidence of infection; (+), localized abscess; (++), localized abscess 2 cm or more in diameter; (+++), localized abscess, necrotic, not fatal; (++++), spreading infection, fatal in 1 to 3 days.

To supply bacteria with a slowly disseminating source of hemin or succinate, a 2% agar solution was mixed with sodium succinate (final concentration of 0.5 M) or hemin (10 μg/ml). The agar was allowed to gel and was then pushed first through a fine nylon mesh and finally through an 18-gauge needle. A 2-ml amount of supplemented agar was mixed with bacteria (2×10^9), and 0.5 ml was injected subcutaneously into the groin of a guinea pig.

Fatty acid determination. Volatile and nonvolatile methylated fatty acids were extracted and analyzed as described by Moore (16). Known standards of volatile and methylated fatty acids were prepared with each set of samples. Column packing material and operating conditions have been detailed elsewhere (14).

Purification of growth factor. Cultures suspected to have growth-promoting activity were tested in the following growth assay. A 10-ml portion of the culture was centrifuged (10 min at 10,000 × *g*), and the supernatant was filter sterilized. The filtrate was then inoculated with a hemin-depleted culture of *B. asaccharolyticus*. Material which was shown to have growth-promoting activity was concentrated (20 times) by freeze-drying.

The rehydrated material was filter sterilized and assayed for growth activity as described earlier, except that 0.5 ml of concentrate was added to 10 ml of a depleted medium before inoculation. Readings of the optical density at 660 nm for both growth assays (before and after freeze-drying) were similar. Fractionation of the concentrated supernatant was done on a precalibrated Sephadex (G-15) column (Pharmacia Canada Ltd). Elution was performed with phosphate buffer (50 mM, pH 6.6). A total of 75 fractions, 2 ml each, were collected and assayed for (i) growth-promoting activity (0.8 ml added to 4 ml of basal medium inoculated with hemin-depleted strain K110) and (ii) volatile and nonvolatile fatty acids by gas chromatography.

Collagenase and protease activity. Collagenase was measured as described by Gisslow and McBride (6). Proteolytic activity was determined by measuring activity against Azocoll (Calbiochem, La Jolla, Calif.) or casein (Hammersten quality). The reaction mixture for the Azocoll assay contained: tris(hydroxymethyl)-aminomethane-hydrochloride buffer (0.05 M, pH 7.2), 4.8 ml; neutralized cysteine hydrochloride in the same buffer (0.05 M), 0.2 ml; and supernatant from a centrifuged culture, 0.5 ml.

The reaction components were preincubated at 37°C for 15 min before the addition of 20 mg of Azocoll. Incubation was then continued at the same temperature in a shaking water bath. Samples (2 ml each) were removed at various time intervals, chilled on ice, and filtered to remove insoluble substrate. The amount of dye released was determined by measuring the absorbance of the filtrate at 520 nm.

The substrate for the casein assay was prepared by dissolving 1 g of casein in 100 ml of phosphate buffer (pH 7.4) and treating for 15 min in a boiling water bath. The reaction mixture contained 2.5 ml of casein, 1.0 ml of neutralized cysteine hydrochloride (0.05 M),

0.5 ml of supernatant from a sedimented culture or 100 μl of a 10× concentrated cell suspension, and buffer to 5 ml. The mixture was incubated at 37°C, and the reaction was terminated by the addition of 5.0 ml of 10% trichloroacetic acid. After 30 min at room temperature the contents of the tubes were filtered, and the absorbance (280 nm) of the filtrates was determined.

Glucose determination. Glucose was measured by either the anthrone assay (26) or by the peroxidase-glucose oxidase assay (Sigma).

Porphyrin and hemin extraction. Porphyrin and hemin were extracted with ethyl acetate and hydrochloric acid by the one-step procedure described by Falk (2).

Chemicals. The siderochromes rhodotorulic acid, Desferal, and enterobactin were obtained from J. B. Neilands.

RESULTS

Isolation of an infectious consortium. The sulcular flora from a periodontal lesion in a patient with advanced periodontitis was inoculated into the groin of a guinea pig. The following day the animal had developed a large lesion containing a thin, serous, dark-colored, foul-smelling exudate. Inoculation of a second animal with as little as 0.1 ml of this exudate produced an identical, rapidly spreading infection. Autopsy of the animals showed marked tissue necrosis in the abdominal region with perforation of the peritoneal wall in one of the animals. Microscopic analysis of the exudate revealed a variety of gram-negative and gram-positive cocci and rods. The exudate from the second animal was serially diluted under anaerobic conditions and plated on blood agar, which was incubated anaerobically. All colonies on the high dilution plates were isolated in pure culture. Analysis of the isolates showed that there were six different organisms, *B. asaccharolyticus* (GP-14) and five others, that could be distinguished on the basis of cellular morphology and Gram stain. A mixture of the five organisms was not infective unless it was combined with *B. asaccharolyticus*.

To establish the minimum number of species capable of initiating an infection, guinea pigs were inoculated with *B. asaccharolyticus* strain K110 and various combinations of the other five strains. Cells for inoculum were harvested from surface growth on laked blood agar plates that had been streaked and incubated anaerobically for 4 days. Cells were suspended in phosphate-buffered saline + thioglycolate. All possible combinations of *B. asaccharolyticus* and 1, 2, 3, or 4 of the other strains were tested. Four guinea pigs were inoculated with each combination. Infections were established only when both *B. asaccharolyticus* and *K. pneumoniae* (strain 20) were included in the inoculum. Neither *B. asaccharolyticus* nor *K. pneumoniae* alone could produce an infection. The other organisms tested appeared to have no effect on the severity or establishment of infection.

Four other oral isolates of *B. asaccharolyticus* (two from dogs, two from humans) were examined to determine whether they were infective when injected together with *K. pneumoniae*. None was infective alone, but all four were infective when combined with *K. pneumoniae*.

The possibility that the nature of the synergic effect might be the result of interactions not requiring bacterial growth was also investigated. *B. asaccharolyticus* strain K110 was killed by vigorously aerating the cells and *K. pneumoniae* by exposure to $CHCl_3$. In the latter case $CHCl_3$ was removed by prolonged sparging with air at 37°C. The effectiveness of the killing procedures was assessed by plating the organisms on blood agar. Both microorganisms had to be alive to successfully infect an animal. It was also found that a sterilized exudate prepared from an infected guinea pig did not cause abscess formation.

Number and proportion of cells required to initiate an infection. Cells of strain K110 and *K. pneumoniae* were grown for 48 h, and total numbers were determined by using a Petroff-Hausser chamber. They were then mixed and injected into guinea pigs. When equal numbers of bacteria were mixed, the minimum total number of bacteria required to produce an infection was approximately 10^8. However, the most important information obtained from this experiment relates to the nature of the infection induced by different numbers of cells. When smaller numbers were injected (2.5×10^8 total) the lesion was slow to develop and invariably became localized (<2 cm), whereas injection of larger numbers of cells (4×10^8 total) caused the more acute, rapidly spreading form of the disease. The effect of varying the proportions of cells in the infectious mixture was also investigated to determine optimum concentrations and threshold values. The minimum number of *Bacteroides* necessary to initiate a disease process was 10^8 organisms but only when combined with 7.5×10^8 cells of *K. pneumoniae*. Any reduction in the number of *Klebsiella* yielded a noninfectious mixture. The infection became more severe as the number of *Klebsiella* was increased from 7.5×10^8 to 1×10^9. Increasing the number of *K. pneumoniae* cells beyond 1×10^9 did not increase the pathogenicity of the mixture.

Evidence for nutritional dependence. Preliminary experiments using hemin-free solidified medium indicated that *K. pneumoniae*, *E. coli*, or *A. viscosus* could supplement the growth of

strain K110. Although the experiments did not show that the factor was hemin, it pointed out the nutritional dependency of *B. asaccharolyticus*. To identify the growth factor(s), it was necessary to formulate a liquid medium which could (i) duplicate the plate phenomenon described above, (ii) support the growth of both organisms when specific components were added, and (iii) serve for subsequent analysis of the growth factor(s). Nutritional studies showed that a basal medium supplemented with 0.1% glucose could fulfill the conditions.

The influence of *Klebsiella* on the growth of the *Bacteroides* was investigated in the glucose-supplemented basal medium (Fig. 1). There was no growth in the medium inoculated with *B. asaccharolyticus*. In pure cultures of *K. pneumoniae*, glucose could not be detected in the medium after 12 h, indicating that glucose was growth-limiting for this species. A diphasic growth curve was noted in medium inoculated with both organisms. The initial increase in absorbance corresponded to the growth of *K. pneumoniae*. The second growth phase was shown to result from the growth of *B. asaccharolyticus*, since two enzyme activities associated with strain K110 (collagenase and protease) appeared in the second half of the diphasic curve. In addition and concomitantly, both the fatty acids detected and the plate counts indicated that *B. asaccharolyticus* was growing. Before the second growth phase it was not possible to detect butyric or phenylacetic acid, both of which are produced during growth of *B. asaccharolyticus* (14).

The nutritional dependency of the *B. asaccharolyticus* on the *Klebsiella* could also be

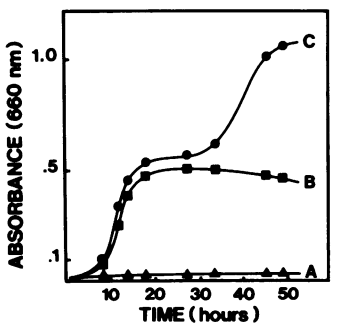

FIG. 1. *Co-cultivation of K. pneumoniae and B. asaccharolyticus. The Bacteroides inoculum was grown in hemin-free medium for 48 h, and the Klebsiella inoculum was taken from a 12-h culture grown in Trypticase soy broth. Both cultures were adjusted to an absorbance at 660 nm of 1.0 and inoculated into basal medium supplemented with glucose (0.1%). (A) B. asaccharolyticus strain K110; (B) K. pneumoniae; (C) strain K110 plus K. pneumoniae.*

demonstrated when a hemin-depleted culture of K110 was added to a sterile culture filtrate from *K. pneumoniae*. The *Klebsiella* was grown in glucose (0.1%)-supplemented basal medium. At various times after inoculation, a 10-ml portion of the culture was removed and filter sterilized. The filtrate was then inoculated with hemin-depleted strain K110, and growth was measured after 24 and 48 h. The results confirmed the evidence that *Klebsiella* produced a growth-stimulatory factor for strain K110 and that the factor was produced as early as mid-logarithmic growth phase. A similar experiment using *K. pneumoniae* as inoculum and strain K110 culture filtrate showed that growth of the facultative organisms was not enhanced by strain K110.

Identification of the growth factor. The growth factor was stable indefinitely at 37°C, stable to autoclaving, not oxygen sensitive, and passed through a UM-05 ultrafiltration membrane (Amicon Corp., Lexington, Mass.), indicating a molecular weight of less than 500. Batch treatment of the concentrated filtrate with ion exchange resins indicated that the compound was negatively charged.

Attempts to extract hemin or porphyrin-like molecules from sterile culture filtrates of *K. pneumoniae* were unsuccessful. Similarly, it was not possible to replace the growth factor by supplementing the basal medium with iron-chelating molecules such as Desferal, rhodotorulic acid, enterobactin, and ferrichrome in various concentrations (100, 10, and 1 μg/ml). These results are in agreement with the fact that these types of compounds are not synthesized under anaerobic conditions.

Since the factor supplied by *K. pneumoniae* was not hemin or a porphyrin derivative and presumably not a protein (boiling and autoclaving resistant), it was decided to analyze the culture supernatant for metabolic end products. *Klebsiella* grown anaerobically in glucose-supplemented (0.1%) basal medium was found to produce large amounts of succinic acid. At the onset of the stationary growth phase the succinic acid concentration was approximately 600 μg/ml; metabolism continued in the absence of an increase in cell numbers, and by 48 h the succinic acid concentration had reached 919 μg/ml. This was of particular interest because of the known stimulatory effect of succinate on the growth of the pigmented rumen *Bacteroides* (9). These results supported the idea that succinic acid was a potential growth factor. The possibility was examined by inoculating a succinate-supplemented, hemin-free basal medium with a hemin-depleted inoculum of *B. asaccharolyticus* strain K110 (Fig. 2). It is clear that succinic acid can replace hemin, although the amount of

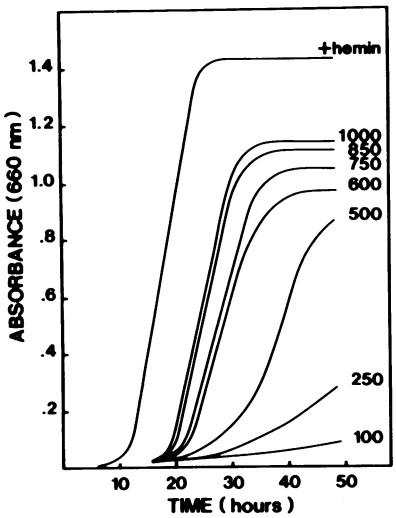

FIG. 2. *Growth characteristics of B. asaccharolyticus strain K110 in succinate- or hemin-supplemented medium. Basal medium supplemented with hemin (5 × 10^{-3} g·liter^{-1}) (curve labeled + hemin) and basal medium supplemented with succinate (micrograms per milliliter added shown for each growth curve) were inoculated with a 48-h culture of hemin-depleted strain K110 and incubated for 48 h at 37°C. At various times, absorbance at 660 nm was read for each culture.*

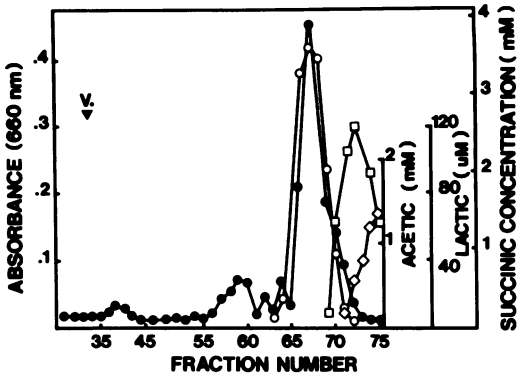

FIG. 3. *Fractionation of the K. pneumoniae culture filtration on Sephadex G-15. The growth factor was obtained from 200 ml of a 10-h culture of anaerobically grown Klebsiella on glucose (0.1%)-supplemented basal medium. The culture supernatant was concentrated 20 times by freeze-drying. A 5-ml amount of concentrated culture filtrate was applied to a Sephadex G-15 column (2.6 by 40 cm) and eluted with phosphate buffer (50 mM, pH 6.6). Each fraction was assayed for volatile and nonvolatile fatty acids and for growth-promoting activity. Symbols: ●, absorbance at 660 nm; ○, succinic acid; □, lactic acid; ◇, acetic acid.*

succinic acid required for strain K110 to reach maximum optical density is high. This suggests that succinate might be used as an energy source rather than as a precursor for heme biosynthesis. The growth rate in the presence of 750, 850, and 1,000 μg of succinate or hemin per ml is identical. However, for all the succinate concentrations assayed, growth was preceded by a longer lag period than was observed in the presence of hemin. The lag period of 10 to 15 h was also found when mixed cultures of the two organisms were grown in glucose-supplemented medium (see Fig. 1). A procedure employing gel filtration was used to isolate the growth factor produced by *K. pneumoniae*. Growth stimulation was confined to a single, symmetrical peak which corresponded to the position where succinic acid eluted from the column (Fig. 3). There was excellent correlation between the amount of succinic acid present in the fraction and the amount of growth stimulation. Acetic and lactic acids were detected in the eluant but they were well separated from the growth factor.

Growth stimulation by other bacteria and in vivo studies. The relationship between endogenously produced succinate and the growth of the *Bacteroides* was evaluated further by assessing (i) the ability of a number of organisms to stimulate growth of strain K110 and (ii) the success of mixtures in the animal model. The results (Table 1) show that only organisms which produce succinic acid stimulate the growth of strain K110. The growth factor produced by *E. coli* B23 was also characterized by the procedures described for *Klebsiella*. The compound has similar properties and eluted at the same position from a Sephadex G-15 column.

These succinate-producing organisms were also capable of forming an infective consortium with strain K110. None was infective when inoculated in pure culture. Both *E. coli* and *A. viscosus* supported the development of the rapidly spreading fatal form of the disease, whereas *B. melaninogenicus* OBM-3 led to formation of a localized, pustular abscess. The bacteria that did not produce succinic acid were not infective when injected along with strain K110.

Further proof that succinic acid is the factor required to induce formation of a pathogenic lesion was obtained from studies in which pure cultures of *B. asaccharolyticus* strain K110 were inoculated along with succinate. In the initial experiments cells were mixed with hemin or succinate and injected into the animal, but unfortunately lesions did not form. A possible explanation for this lack of success is that the growth factor diffuses away from the injection site before *B. asaccharolyticus* begins growing.

An attempt was made to slow this diffusion by incorporating the growth factor into an agar matrix. When this was done, pure cultures of the

TABLE 1. *Effect of other types of bacteria on growth and infectivity of B. asaccharolyticus K110*

Bacterium	Succinate production[a]	Growth stimulation[b]	Infectivity[c]	Infectivity with *B. asaccharolyticus* K110[d]				
				1[e]	2	3	4	10
K. pneumoniae	+	+	−	+++	Dead			
E. coli B23	+	+	−	+++	Dead			
A. viscosus	+	+	−	++	+++	Dead		
B. melaninogenicus OBM-3	+	+	−	+	++	++	++	+
F. nucleatum	−	−	−	−	−	−	−	−
S. sanguis	−	−	−	−	−	−	−	−
S. albus	−	−	−	−	−	−	−	−
Veillonella sp.	−	−	−	−	−	−	−	−

[a] As determined by gas-liquid chromatography.
[b] As determined by growth in filtrate assay and cocultivation assay.
[c] 10^9 cells injected. Two animals were tested per bacterium.
[d] Number of cells injected: 1×10^9; e.g., 5×10^8 cells of each of the two bacteria. Two animals were tested per mixture of bacteria.
[e] Days after infection.

organism were found to be infective. Hemin supported development of the rapidly spreading, fatal disease, and succinate supported development of the less severe, localized disease. This is in keeping with the in vitro studies which showed that the lag period was longer when strain K110 was grown with succinate than with hemin.

DISCUSSION

To understand any mixed infection presupposes that such a bacterial mixture contains the minimum number of species required to produce the disease. The first requirement is to delineate the species involved in the disease from the "accidental" ones. The more simple the system, the easier it is to study the interactions among the organisms involved.

In contrast to the findings of other workers (12, 24), our studies have shown that it is possible to obtain a simple two organism system. *B. asaccharolyticus* played an essential role in the disease. Similar conclusions have been reached recently by Slots and Hausmann (20). Our studies also suggested that the "helper" organism did not possess important pathogenic properties. The result showed that the successful mixture consisted of *B. asaccharolyticus* and *K. pneumoniae*.

The characteristics of the transmissible, mixed infection produced by the injection of guinea pigs with organisms cultured originally from gingival material were similar to those described by MacDonald and co-workers (10). The relation between the two bacteria was not restricted to this pair, since a number of other combinations involving different strains of *B. asaccharolyticus* were effective. The reverse situation where the helper organism was interchanged was also found to be successful. Quantitative studies showed that high numbers of *B. asaccharolyticus* were required along with *K. pneumoniae* to produce an infection. When this requirement was met, the results indicated that increasing numbers of *K. pneumoniae* increased the severity of the infection. Two reasons might explain the fact that large numbers of cells are required to cause an infection in the animal system: (i) low-level pathogenicity and (ii) sensitivity to oxygen present at the newly infected site.

The requirement was identified as succinic acid, which was able to replace hemin in the growth medium. The succinate was needed in relatively high concentration to promote the growth of *B. asaccharolyticus*. The results confirmed similar studies done by Lev et al. (9) on a rumen strain of *B. melaninogenicus*. However, the role of succinate has not yet been defined. Preliminary experiments with labeled succinate have indicated that the compound might be used as an energy source or an electron sink, since very little or no succinate was incorporated into cell carbon.

Experiments with mixed cultures thus show that a food chain exists in the system studied. Glucose is metabolized by *K. pneumoniae* under anaerobic conditions to produce large amounts of succinic acid. The acid is then consumed by *B. asaccharolyticus* to support its growth.

The nutritional dependency of strain K110 was also shown when various organisms producing succinate were found to enhance its growth in vitro and in the infectivity assay, whereas organisms not producing succinic acid gave negative results in all assays. The positive results obtained are also not likely to be due to endotoxic activity from the helper organisms, since positive results were obtained when gram-positive, succinate-producing organisms were coin-

jected with *B. asaccharolyticus* strain K110.

The most important and definitive evidence indicating the nutritional dependency was obtained by infecting animals with pure cultures of strain K110 together with a slowly diffusing source of succinate or hemin. The experiment eliminates the possibility that in the model system the critical function of the "helper" organism is to reduce oxygen tension. In this assay large numbers of cells, many of them present as clumps, probably provide sufficient protection against oxygen. However, in a natural infection where fewer organisms are involved the second organism may facilitate the expression of pathogenicity by creating an anaerobic environment in addition to supplying succinate.

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