Isolation of colonial variants of *Bacteroides gingivalis* W50 with a reduced virulence

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Summary. The spontaneous appearance of unusual colony forms was observed during prolonged growth of *Bacteroides gingivalis* W50 in a chemostat. Two variants were selected for further study which could be distinguished from the parent strain by the rate and intensity of pigmentation of their colonies. For example, after anaerobic incubation for 14 days, variant W50/BR1 produced brown colonies whereas those of the parent strain were black; in contrast, variant W50/BE1 did not show signs of pigmentation until incubation had continued for 21 days. In subsequent studies in the chemostat, variant W50/BE1 bred true even after prolonged growth whereas other colony forms appeared after incubation of variant W50/BR1 for 14 days. The relatedness of W50/BR1 and W50/BE1 to the parent strain was confirmed by comparisons of the whole-cell fatty-acid profiles, the patterns of pre-formed enzymes and by the metabolic end products after growth. However, the variants did differ from the parent strain in their virulence in a mouse pathogenicity model. The parent strain killed all mice given infective doses $> 5 \times 10^8$ cfu whereas W50/BR1 was much less virulent (2 out of 10 mice killed and higher infective doses needed for higher mortality rates) and W50/BE1 was avirulent at all infective doses tested.

Introduction

Bacteroides gingivalis, a member of the black-pigmented group of *Bacteroides*, has been implicated as a major pathogen in the aetiology of some human periodontal diseases, particularly chronic inflammatory periodontal disease in adults (Slots, 1982a). Representative strains of this species have also been shown to be highly virulent in experimental animal infections (van Steenbergen *et al.*, 1982), although recently this virulence has been shown to depend on the haemin concentration of the growth environment (McKee *et al.*, 1986). *B. gingivalis* produces a wide range of factors that might contribute to its pathogenicity. These include the abilities to (i) damage host tissues directly through the production of enzymes and cytotoxic metabolites, (ii) damage host tissues indirectly by inducing an inflammatory response, and (iii) evade certain host defences by capsule formation and protease production (Slots and Genco, 1984). To determine the relative significance of individual factors, mutants defective in specific traits would be of

particular value. During the prolonged growth of *B. gingivalis* W50 in a chemostat, unusual colony forms were observed in low numbers when small volumes of the culture were plated on to blood-agar plates. These colonies produced pigment only weakly, but the detection of key chemotaxonomic markers established their relatedness to the parent strain. The colonial variants were then compared in an animal pathogenicity test.

Materials and methods

Bacterial strains

The bacteria used in this study were *B. gingivalis* W50, *B. asaccharolyticus* B536 and *B. intermedius* T588 (kindly provided by Dr H. Shah, The London Hospital Medical College, London), *B. endodontalis* HG370 (kindly provided by Dr T. J. M. van Steenbergen, Vrije Universiteit, Amsterdam), and *B. fragilis* NCTC 9344. The bacteria were maintained on blood agar and stored by lyophilisation.

Growth of B. gingivalis in the chemostat

B. gingivalis W50 was grown in an LH series II modular chemostat (LH Engineering, Stoke Poges) as described

previously (McKee *et al.*, 1986). Briefly, cells were grown at a constant pH of 7.5 ± 0.2 and a dilution rate of $0.1 h^{-1}$ (this corresponds to a constant mean generation time of 6·9h). The temperature for growth was 36°C and the culture was gassed continuously with CO_2 5%, $N_2$95%. A modified complex medium (BM), supplemented with haemin 2·5 mg/L but no vitamin K_1 was used as before to give conditions of haemin excess. The chemostat was inoculated and monitored regularly for pH, Eh, turbidity and dry weight of bacteria as described previously (McKee *et al.*, 1986).

Viable counts and recognition of colonial variants

Viable counts were determined at frequent intervals to assess the purity and yield of the chemostat cultures. Culture fluid was serially diluted in BM broth and 100 μl of each dilution was plated on to 10% v/v horse-blood-agar plates (Blood Agar Base no 2, Oxoid). Plates were incubated at 37°C for up to 21 days in an atmosphere of CO_2 10%, H_2 10%, N_2 80%. On these plates colonies with a reduced intensity of pigmentation were recognised. These colonies were sub-cultured, freeze-dried, and grown in the chemostat as described for the parent W50 strain.

Identification of colonial variants

The identity of the colonial variants was assessed by comparing (a) their ability to make acid from glucose-(0·5% w/v)-supplemented BM broth, pH 7·0, (b) the presence of pre-formed enzymes in the API ZYM (API, Basingstoke) system, (c) their whole-cell fatty-acid profiles, and (d) their pattern of fermentation products, with those of the parent W50 strain. The API ZYM system was used according to the manufacturer's instructions. Fermentation products were measured in culture supernates by standard gas-liquid chromatographic techniques. Briefly, volatile fatty acids were quantified with a Pye GCV gas chromatograph fitted with a flame-ionisation detector and a PU 4800 video chromatography control centre for integration; the internal standard was 2-ethyl butyric acid. The column (2 m × 4 mm) was packed with Chromosorb W (80–100 mesh) coated with carbowax 20M 15% and phosphoric acid 2% (Chrompack, Middleburg, Holland); the carrier gas was nitrogen (flow rate 20 ml/min). The initial oven temperature was 90°C and was raised to 118°C at 4°C/min; the temperatures of the injector and the detector were 250°C and 260°C, respectively. The presence of phenylacetic acid was confirmed by comparison with a standard by mass spectrometry with a Dupont 21–491 instrument operated in the electron impact mode at 70 eV.

The fatty-acid profiles of cells grown for 10 days on blood-agar plates were determined as described by Wait and Hudson (1985). Cells were freeze-dried, suspended in 1 ml of toluene:methanol (1:1), acidified with 25 μl of concentrated sulphuric acid and incubated overnight at 60°C. Samples were cooled, 0·5 ml of saturated sodium chloride solution was added, and the fatty acid methyl esters were extracted with two successive 1·5-ml volumes of hexane:chloroform (4:1). The extracts were concentrated to one-third volume under nitrogen, washed with 1 ml of 0·3 M NaOH, and the organic layer was removed and evaporated to dryness. Trimethyl-silyl ethers were prepared by treating the methyl ester with bis-(trimethylsilyl)trifluoroacetamide (Fluorchem, Glossop) 20% in pyridine. Fatty acids were analysed by capillary gas-liquid chromatography in a Carlo-Erba 4130 chromatograph (Carlo-Erba, Milan, Italy), fitted with a 12-m vitreous silica column coated with BP-1 (SGE Ltd, Milton Keynes). Helium was the carrier gas (flow rate 1 ml/min), and samples were introduced by splitless injection in *iso*-octane. The initial oven temperature was 80°C, and was raised to 195°C at 40°C/min after a 1-min delay. Peak identities were established by comparison with standards and by gas-liquid chromatography-mass spectrometry, by use of a Kratos analytical MS80 RFA (Kratos Ltd, Manchester) interfaced to a gas-liquid chromatograph (Carlo-Erba, Milan, Italy). Chromatographic conditions were as described above, and the mass spectrometer was operated at a scan rate of 0·3 s/decade of mass in electron inpact mode at an ionising energy of 70 eV and 100 μA trap current.

Mouse pathogenicity test

The virulence of the colonial variants was compared with that of the parent strain in a pathogenicity model based on that of van Steenbergen *et al.* (1982). Bacteria were grown in haemin-excess conditions in the chemostat and injected subcutaneously (s.c.) into mice as described previously (McKee *et al.*, 1986). Viable counts were performed on the inocula to establish the cell dosage. Mice were killed with ether at various stages after infection. Samples of infected tissues taken at necroscopy were fixed in buffered neutral formalin 10% v/v and embedded in paraffin wax by standard procedures. Sections of these blocks were cut at 5-μm intervals and stained with haematoxylin and eosin.

Results

Appearance of colonial variants of B. gingivalis W50

During the growth of *B. gingivalis* W50 in conditions of haemin excess in a chemostat at pH 7·5, small volumes of culture were plated on to blood agar for viable count determinations and to check the purity of the culture. After growth for 2–3 weeks in a chemostat (49–73 bacterial generations), colonies with atypical morphology were observed. These colonies differed from the majority in the intensity of their pigmentation (table I). After anaerobic incubation for 14 days on plates, the colonies of one variant (W50/BE1) were beige,

Table I. Appearance of colonial variants during growth of *B. gingivalis* W50 in a chemostat*

Colony type	Pigmentation on blood agar after		Approximate percentage† of colonies from chemostat culture after		
	14 days	21 days	7 days	14 days	21 days
Parent, W50	black, with tan centre	black	93	88	66
W50/BR1	brown, with clear edge	dark brown	1	7	10
W50/BE1	beige	tan–dark tan	6	5	24

*Cells were grown under haemin excess, pH 7.5 ± 0.2, at a constant mean generation time of 6·9h.
† Viable counts were taken from a single chemostat run; similar trends were found with other chemostat experiments.

while those of another were brown (W50/BR1). These colonial variants were only a small percentage of the culture, until after growth for 21 days in the chemostat when the proportions of variant W50/BE1 could reach, on occasions, c. 24% of the total colony forming units (table I).

Stability of growth of colonial variants on sub-culture

An example of each of the two variants was sub-cultured on blood agar and found to breed true. Chemostats were also inoculated with each variant and the cultures were grown in identical conditions to those used originally for the parent W50 strain. A chemostat inoculated with W50/BE1 yielded only beige colony forms even after prolonged incubation; no black or other strongly-pigmenting colonies were ever observed. In contrast, when variant W50/BR1 was grown in the chemostat, other weakly-pigmenting colonies were detected although these were <10% of the total colony types during incubation up to 14 days, but increasing to nearly 30% of the total colony types after growth for 36 days. All three strains were asaccharolytic, being unable to lower the pH below 7·0 when inoculated into BM broth supplemented with glucose.

Characterisation of the colonial variants

To determine the relatedness of the variants to the parent strain, comparisons were made of the patterns of fermentation products, the long chain fatty-acid profiles of whole cells, and the presence of key enzymes in a semi-quantitative assay. The fermentation products are listed in table II. The

same pattern of acids was produced by all three strains, although low concentrations of *iso*-caproic and caproic acid were detected only in supernates of strain W50/BR1. All three strains produced similar amounts of phenylacetic acid.

The whole-cell fatty acids of *Bacteroides* spp. are useful chemotaxonomic markers (Shah and Collins, 1980). Consequently, the profiles of whole cells of the variants were compared with those of W50 and other asaccharolytic *Bacteroides* spp. which might be confused with *B. gingivalis* (table III). There was a stronger similarity between the fatty-acid profiles of the variants and the parent W50 strain than between these strains and the other *Bacteroides* spp.

Table II. Acid fermentation products of the parent strain and two colonial variants of *B. gingivalis* W50

Fermentation product	Amount* of product (μmol/mg dry wt of cells)		
	W50	W50/BR1	W50/BE1
Acetic acid	14·1 (0·14)	9·7 (2·3)	13·6 (1·8)
Propionic acid	1·9 (0·6)	1·8 (0·6)	1·7 (0·3)
Iso-butyric acid	3·2 (1·0)	4·3 (0·7)	3·7 (0·9)
Butyric acid	16·9 (2·1)	16·6 (1·8)	11·8 (1·1)
Iso-valeric acid	7·2 (0·9)	10·2 (0·6)	5·8 (0·7)
Valeric acid	<0·1	0·4 (0·5)	0·3 (0·3)
Iso-caproic acid	ND	0·5 (0·4)	ND
Caproic acid	ND	0·8 (0·4)	ND
Succinic acid	0·2 (0·1)	0·9 (0·2)	0·2 (0·02)
Phenylacetic acid	1·5 (0·6)	1·6 (0·7)	1·5 (0·8)
Hydrocinnamic acid	<0·1	<0·1	<0·1

*Mean of at least three determinations (SD) made on supernates taken on different days from the chemostat.
ND = not detected.

Table III. Profiles of the major long chain fatty acids of whole cells* of colonial variants of B. gingivalis W50 and other Bacteroides spp.

Species and strain	Percentage fatty-acid composition						Ratio i-15: ai-15	Total hydroxy-fatty acid
	i-15	ai-15	3(OH)15	16:0	3(OH)i-17	18:2		
B. gingivalis W50	30	7	5	10	30	3	4·3	40
B. gingivalis W50/BR1	23	6	5	8	39	5	4·1	49
B. gingivalis W50/BE1	19	4	4	10	44	3	4·8	52
B. endodontalis HG370	47	3	7	14	12	2	15·7	21
B. asaccharolyticus B536	58	1	5	7	4	3	58·0	9
B. intermedius T588	12	13	5	5	20	15	0·9	27
B. fragilis NCTC 9344	13	23	3	7	24	3	0·6	34

* Cells were grown on blood-agar plates.
i-15 = 13-methyltetradecanoic acid; ai-15 = 12-methyltetradecanoic acid; 3(OH)-15 = 3 hydroxy-13-methyl tetradecanoic acid; 16:0 = hexadecanoic acid; 3(OH)i-17 = 3-hydroxy-15 methyl hexadecanoic acid; 18:2 = ocatadeca-dienoic acid.

This was particularly marked when the ratios of i-15:ai-15 and the total hydroxy-fatty acid contents were compared (table III).

When the enzyme activities of the B. gingivalis strains were compared in the API-ZYM standard galleries, all three colony types gave similar profiles—they gave positive results for acid and alkaline phosphatases, C_4 and C_8 esterases, trypsin, phosphoamidase and N-acetyl-β-glucoaminidase. One consistent difference, however, was that variant W50/BE1 had only weak trypsin activity, whereas both W50 and W50/BR1 gave strongly positive reactions.

Pathogenicity of colonial variants of B. gingivalis

B. gingivalis W50 is highly virulent when injected s.c. into mice, although this virulence is dose-dependent (McKee et al., 1986). Marked differences in disease-producing potential were found when the colonial variants were compared with the parent strain in this simple mouse pathogenicity model (table IV). Both variants were less virulent than W50. At a dose of 1×10^9 viable cells, strains W50/BE1, W50/BR1 and W50 killed 0, 2 and 10 out of groups of 10 mice, respectively. The mortality rate with W50/BR1 increased when higher numbers were injected but W50/BE1 remained avirulent irrespective of the dose.

Pathology of lesions

In all the mice inoculated with B. gingivalis W50 there was spreading inflammation involving the skin of the lower flanks and ventral abdomen. Large areas of skin became hairless, dark brown and scabbed, and on dissection the subcutaneous tissues were distended by a reddish-brown fluid. Histological examination showed extensive exudative and necrotising changes from the epidermis to the abdominal muscle layers, with some localised abscess formation and marked diffuse accumulation of polymorphonuclear leucocytes (PMN) and oedema. Animals given a dose of $\log_{10}$ 9·3 viable cells of variant W50/BR1 had abdominal skin lesions which were grossly and microscopically similar to those produced by the parent strain. Animals which survived after having been given a smaller dose of W50/BR1 (table IV) had no macroscopic lesions, but histologically there were foci of inflammation

Table IV. Pathogenicity in mice of colonial variants of B. gingivalis*

Colony type	Subcutaneous infective dose ($\log_{10}$ cfu)	Number of mouse deaths/total number of mice challenged
Parent W50	9·3	10/10
	9·0	10/10
	8·7	10/10
	7·7	0/10
Variant W50/BR1	9·3	8/10
	9·2	2/10
	9·0	2/10
	8·9	0/10
Variant W50/BE1	9·6	0/10
	9·3	0/10
	9·1	0/10

*Bacteria were grown in a chemostat at a dilution rate of $0·1$ h^{-1} (mean growth rate 6·9 h) and at constant pH 7·5 in haemin excess conditions. Pathogenicity tests were repeated on different days at various doses with freshly-prepared cells.

(PMN and histiocytes) in the dermis. Three of the ten mice inoculated with $\log_{10}$ 9·6 viable cells of variant W50/BE1 had slight localised scab formation on the abdominal skin. Histologically, these animals showed areas of inflammatory changes in the dermis characterised by oedema, PMN infiltration and histiocyte activity, though these changes were not as severe or extensive as with W50 or W50/BR1. The remaining mice and those given a lower infective dose had no macroscopic lesions, and only a few foci of mild dermal inflammation were observed microscopically.

Discussion

The isolation of spontaneous variants (isogeneic mutants) with reduced virulence has enabled properties to be recognised which contribute to the pathogenicity of several gram-negative bacteria (Birkbeck and Penn, 1986). Generally, these variants have been identified by a fortuitous association of the loss of virulence with either an altered colony appearance or a change in the ability of an organism to grow on certain media. For example, Bordetella pertussis can spontaneously and irreversibly lose virulence factors in a process termed phase variation (Leslie and Gardner, 1931) and the colonial morphology of avirulent forms (corresponding to phases III and IV) are different from those of virulent B. pertussis on the same medium (Peppler, 1982; Peppler and Schrumpf, 1984). Similarly, gonococci can produce a number of colonial variants in vitro (Kellogg et al., 1963) and during natural infection (Schwalbe et al., 1985) that differ in the expression of pili and certain outer membrane proteins (Heckels, 1986). These gonococcal variants also differ in their attachment to host surfaces and their ability to undergo antigenic variation may enable cells to evade host defences. Recently, atypical translucent colony forms of Vibrio vulnificus were isolated and all were found to be avirulent and to have lost certain virulence factors (Simpson et al., 1987).

In the present study, atypical colony forms were observed when cultures of B. gingivalis were plated on to blood agar. Characteristically, B. gingivalis produces black colonies but these colonial variants were recognised by their weak pigmentation. Indeed, variant W50/BE1 exhibited no pigmentation even after incubation for 14 days and colonies became only slightly pigmented after incubation for 21 days. Colonies of this latter variant were originally considered to be most likely those of a contaminant. However, when the properties of W50/BE1 and another variant (W50/BR1) were

studied in more detail, both had a phenotype similar to that of B. gingivalis W50. In particular, both variants were asaccharolytic and produced a wide range of metabolic end products, with acetic and butyric as major acids, which is characteristic of Bacteroides spp. (Holdeman et al., 1977), and large amounts of phenylacetic acid which distinguishes B. gingivalis from other asaccharolytic black-pigmented Bacteroides (Kaczmarek and Coykendall, 1980; Ferguson et al., 1983). Whole-cell fatty-acid patterns have been suggested to be of great value in differentiating Bacteroides spp. (Shah and Collins, 1980). Again, the two variants had profiles similar to the parent strain, and all three showed striking differences from other asaccharolytic and related Bacteroides spp. with which they might have been confused. Furthermore, all three strains gave similar reaction patterns in the API ZYM rapid enzyme identification system, and these were consistent with previously published profiles of B. gingivalis strains (Slots, 1982b; Laughon et al., 1982), with the exception that variant W50/BE1 had only weakly positive trypsin activity. Nevertheless, the collective evidence is consistent in showing that the variants are closely related to B. gingivalis and are, therefore, probably spontaneous isogeneic mutants of strain W50.

A major difference was found when the virulence of the variants was compared in a simple mouse pathogenicity model. In the absence of a suitable animal model of periodontal disease, this system has enabled comparisons to be made of the pathogenic potential of different strains and species of oral Bacteroides (van Steenbergen et al., 1982, 1987; McKee et al., 1986). Previous studies (van Steenbergen et al., 1982; McKee et al., 1986), as well as the work presented here, had shown that B. gingivalis strain W50 was highly virulent in mice, causing a fatal infection with widespread exudative and necrotising lesions. Variant W50/BR1 was less virulent than the parent strain and required a higher infective dose to produce similar lesions; variant W50/BE1 was avirulent at all doses tested.

Bacteroides gingivalis produces a range of factors that may enable the organism to evade the host defences and cause tissue damage directly (Slots and Genco, 1984). A comparison of the putative virulence factors produced by the parent strain with those of the variants (and particularly strain W50/BE1) might enable progress to be made in determining the relative importance of individual factors, in a similar manner to that used with variants and mutants of other species such as B. pertussis (Robinson et al., 1986) and N. gonorrhoeae (Heckels, 1986). It is not known yet whether other

strains of *B. gingivalis* show a similar tendency to produce variants. The widespread ability of strains to produce variants might indicate a strategy by which *B. gingivalis* could persist during an infection, evading host defences or adapting to an ever-changing host environment, as has been suggested for the gonococcus (Heckels, 1986).

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