Avirulence of 27 Bacteria That Are Budding, Prosthecate, or Both

O. FAMUREWA,[†] H. G. SONNTAG,[†] AND P. HIRSCH*

Institut für Allgemeine Mikrobiologie, Institut für Hygiene und medizinische Mikrobiologie, Universität Kiel, Kiel, Federal Republic of Germany

We tested the possible pathogenicity of 27 bacteria that are budding or prosthecate or both in mice and guinea pigs. The strains tested belong to the following taxa: *Hyphomicrobium* (12 strains), "*Hyphomonas*" (4 strains), *Pedomicrobium* (2 strains), *Rhodomicrobium* (2 strains), *Prosthecomicrobium* (1 strain), "*Stella*" (1 strain), *Blastobacter* (2 strains), *Planctomyces* (1 strain), and *Pasteuria* (1 strain); 1 strain was unnamed. Mice injected intravenously with a 0.5-ml suspension containing up to 10^9 colony-forming units per ml survived for 7 days without macroscopic changes, abscesses, or lesions. Four *Hyphomicrobium* strains, one "*Hyphomonas*" strain, and the unnamed strain caused slight spleen enlargement and in some cases a mottled kidney appearance; however, the organs were histologically normal, and the changes observed were considered to be signs of host reaction rather than pathological changes. Subcutaneous injections also did not result in disease. Two *Rhodomicrobium* strains could be reisolated from mice after 21 days; none of the other strains could be recovered after 7 days. Lethal endotoxic activity was not detected with any of the strains when cell sonic extracts were injected intravenously. All strains caused antibody formation, and agglutination dilution titers ranged from 1:2 to 1:64. Serological cross-reactions with six strains indicated the presence of distinct taxonomic groups. *Hyphomicrobia* from soil cross-reacted with each other but not with *hyphomicrobia* from aquatic sources. Immunosuppressive pretreatment with Natulan did not render the mice more susceptible. Other possible indicators of pathogenicity (i.e., mannitol utilization and β -hemolysis) were not observed in the 27 strains. We concluded that the 27 bacteria tested are nonpathogenic for mice or guinea pigs under the conditions used.

Recently, an increasing number of morphologically unusual bacteria have been isolated in pure culture. Among these, gram-negative bacteria that are budding or prosthecate or both have attracted special attention because of their unique morphologies, life cycles, and physiologies (13-18, 37). The sources of isolation have varied and have included soil (14, 39), freshwater (17, 37), brackish water (17, 18), seawater (1, 22), and even humans (31). Many of these bacteria comprise new species or even genera, and so far only a few have been studied in enough detail to be included in standard determinative bacteriology texts (4).

As physiological properties and differentiation are investigated, high cell yields are processed. However, little, if anything, is known about the pathogenicities of these unusual bacteria. Since most of these bacteria have not been obtained from human or animal hosts, the notion that

these organisms are probably not pathogenic prevails. However, Hurlbert et al. (19), Weckesser et al. (41), and Katz et al. (20) have demonstrated that there are potentially pathogenic cell surface components in phototrophic bacteria. On the other hand, the budding and hyphal gram-negative organism "*Hyphomonas polymorpha*," which was obtained from infectious sinusitis, reportedly is not pathogenic (31).

The aim of this study was to investigate the pathogenicities of 27 new morphologically and physiologically unusual bacteria. Laboratory animals (mice and guinea pigs) were challenged as potential hosts by administering high cell numbers and using different modes of infection. Our results indicate that the strains were not pathogenic for the hosts tested, although host reactions to the bacterial antigens were observed.

MATERIALS AND METHODS

Microorganisms. A total of 27 strains of morphologically unusual bacteria were investigated. According to present taxonomy and nomenclature, these strains

[†] Present address: Hygiene Institut, Universität Heidelberg, 6900 Heidelberg, Federal Republic of Germany.

belong to the genera *Hyphomicrobium* (12 strains), "*Hyphomonas*" (4 strains), *Pedomicrobium* (2 strains), *Rhodomicrobium* (2 strains), *Prosthecomicrobium* (1 strain), "*Stella*" (1 strain), *Blastobacter* (2 strains), *Planctomyces* (1 strain), and *Pasteuria* (1 strain). One strain (IFAM 1128) cannot be affiliated yet with any presently known genus. The names, culture media used, sources of isolation, and donors of these organisms are listed in Table 1.

The pathogenic strains *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 11229 were used as positive controls. All cultures were maintained on agar slants stored at 6°C in the dark.

Animals. White albino mice of the specific-pathogen-free strain BALB/c(H-2^d) and female brown mice

of the specific-pathogen-free strain C3H(H-2^k) were used. These animals were kept in groups of up to 10 on sterilized sawdust in disinfected Macrolon cages (type III; PAG, Essen, Federal Republic of Germany) at about 23°C. They were fed with type 1314 Altromin pellets (Altromin, Lage, Federal Republic of Germany) and water ad libitum.

Guinea pigs (*Cavia* sp.) were kept individually in type III Macrolon cages at 22°C and fed type 3012 Altromin and water ad libitum. The mice weighed between 20 and 40 g and were between 6 and 20 weeks old at the start of the experiment. The guinea pigs weighed 400 to 550 g and were about 16 weeks old. All animals used were bred and supplied by the Institut für Hygiene und medizinische Mikrobiologie, Universität

TABLE 1. Bacterial test strains, origins, culture media, and sources

Strain(s)	Origin and enrichment	Medium ^a	Maximum growth temp (°C)	Reference(s)
<i>Hyphomicrobium</i> spp.				
IFAM B-522, IFAM H-526	Lawn soil; mineral salts	337-M	>37	15
IFAM NQ-521 gr, IFAM MEV-533gr	River water; mineral salts	337-M	>37	25
IFAM I-551, IFAM CO-582	Soil; mineral salts, CO and O ₂	337-M	>37	24
IFAM ZV-580, IFAM ZV-622	Peat soil; mineral salts, methanol	337-M	>37	42
IFAM MC-748, IFAM MC-750	Soil; mineral salts, methanol or methylamine	337-M	>30	24
IFAM Wi-926	Pond water; mineral salts	337-M	38	Wieczorek ^b
IFAM KB-677	Sewage; mineral salts	337-M	>30	21
<i>Hyphomicrobium neptunium</i>	Seawater; Casitone, yeast extract, Tris buffer, ASW ^d	383	40	22
IFAM LE-670 ^c				
" <i>Hyphomonas polymorpha</i> "	Nasal sinus infection; blood gelatin, 37°C	Nutrient agar	>37	31
IFAM PR-727, IFAM PS-728				
<i>Hyphomonas</i> -like strain IFAM 1325	Baltic Sea brackish water; sample stored in laboratory	PYGV + 25% ASW	>30	17
<i>Pedomicrobium</i> -like strains	Puddle water; mineral salts	PYGV	>30	11; Babinchak ^e
IFAM 868, IFAM 869				
Unnamed strain IFAM 1128	Rock puddle; yeast extract	PYGV + 0.1% yeast extract	36	9
<i>Rhodomicrobium vannielii</i>	Pond water; ethanol, light, anaerobic	391	54	8; Roggentin ^f
IFAM 1093, IFAM 1178 ^T (= ATCC 17100 ^T)				
<i>Prosthecomicrobium</i> sp. strain IFAM 1316	Baltic Sea brackish water; 0.0025% Bacto-Peptone	387	37–39	17
" <i>Stella</i> " sp. strain IFAM 1312	Baltic Sea brackish water; 0.05% casein hydrolysate	PYGV + 25% ASW	38.5–41	17
<i>Blastobacter</i> spp. strains IFAM 1003, IFAM 1005	Lake water; PYGV medium	PYGV	41, 46	Müller ^g
" <i>Planctomyces limnophilus</i> "	Lake water; PYGV medium	PYGV	39	Müller ^g
IFAM 1007				
<i>Pasteuria ramosa</i> IFAM 1009	Lake water; PYGV medium	PYGV	30	Müller ^g

^a For an explanation of the media, see the text.

^b L. Wieczorek, Ph.D. thesis, Universität Kiel, Kiel, Federal Republic of Germany, 1979.

^c According to Moore and Hirsch (4, 27), this strain is a strain of "*Hyphomonas*."

^d Tris, Tris(hydroxymethyl)aminomethane; ASW, artificial seawater.

^e W. Babinchak, personal communication.

^f P. Roggentin, Ph.D. thesis, Universität Kiel, Kiel, Federal Republic of Germany, 1980.

^g M. Müller, Ph.D. thesis, Universität Kiel, Kiel, Federal Republic of Germany, 1978.

Kiel, Kiel, Federal Republic of Germany.

Culture media. The following media were used for cultivation of *Hyphomicrobium* spp.: medium 337-M was prepared by the method of Hirsch and Conti (15) and was supplemented with 3.38 g of methylamine hydrochloride per liter as the C source and an additional N source and only 3 ml of trace element solution; *Hyphomicrobium neptunium* was grown on seawater medium (22).

Medium PYGV for oligotrophs was prepared by the method of Staley (37), and vitamins were added as described by Van Ert and Staley (39). Artificial seawater, which was added to medium PYGV in some cases, was prepared by the method of Lyman and Fleming (23). Medium 391 has been described previously by Pfennig (29) and contained the trace element solution of Pfennig and Lippert (30), as well as 0.5 g of NaHCO_3 per liter. Medium 387 (J. T. Staley, personal communication) contained 1.0 g of yeast extract (Difco Laboratories, Detroit, Mich.), 20 ml of Hutner basal salts, 1.0 g of glucose, and 1,000 ml of distilled water (pH 7.2 after autoclaving).

The 0.1% yeast extract medium (Difco) used for strain IFAM 1128 (F-1) has been described previously (9). Nutrient broth (Difco) and nutrient agar (Difco) were used for the "*Hyphomonas polymorpha*" strains; when they were used for *Staphylococcus aureus*, these media were supplemented with 5% (vol/vol) sheep blood. *Escherichia coli* was grown on China blue-lactose medium (E. Merck AG, Darmstadt, Federal Republic of Germany). To test for mannitol decomposition, we used mannitol-NaCl agar (Oxoid Ltd., London, England). Hemolysis was tested on nutrient agar containing 5% (vol/vol) sheep blood. All other solid media were prepared with 1.5% Bacto-agar (Difco).

Culture conditions. Actively growing cultures were obtained by subculturing agar slants onto 50-ml portions of medium in 125-ml Erlenmeyer flasks, which were then plugged loosely with cotton or ready-made plugs. After visible growth occurred, this procedure was repeated by using a 2 to 5% inoculum from the growing suspension.

The growth of these active cultures was followed by determining the optical density at 650 nm with a model 101 spectrophotometer (Hitachi, Tokyo, Japan) and simultaneously determining viable counts in a homogenized suspension. To do this, the cells were harvested by centrifugation and washed twice in sterile phosphate-buffered saline (PBS) (pH 7.2) containing (per liter) 9.78 g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 2.45 g of KH_2PO_4 , and 4.5 g of NaCl. The washed suspensions were then placed in 100-ml screw-capped bottles containing about 20 g of sterile glass beads (diameter, 3 mm). Suspensions were homogenized by shaking for 1 to 3 min. Portions (1.0 ml) of serial dilutions in the buffer described above were spread onto four parallel plates containing the appropriate medium. For the *Rhodomicrobium* strains, agar shake cultures were prepared instead. Colonies were counted after 2 to 21 days, depending on the strain.

Most cultures were aerated at 30°C in the dark. *Rhodomicrobium vannielii* strains IFAM 1093 and IFAM 1178^T (type strain) were grown anaerobically in tightly sealed screw-capped bottles at 23°C and 1,500 lx.

Microscopy and photography. All cultures were ex-

amined directly with a phase-contrast Photomicroscope II instrument (Zeiss, Oberkochen, Federal Republic of Germany). Agar-coated slides were used for this (17).

Animal assays for pathogenicity. Culture suspensions for the animal assays were grown as described above in 125-ml flasks previously washed with chromosulfuric acid and rinsed with sterile, particle-free, double-distilled water. Active cells in mid-log phase were injected into animals with nontoxic, pyrogen-free insulin syringes and needles. The mice were sedated with ethyl ether (Aether zur Narkose; Hoechst, Frankfurt, Federal Republic of Germany), and the guinea pigs were injected in a stretched position without sedation.

Generally, each mouse received intravenously in the tail 0.5 ml of a bacterial suspension containing 10^3 , 10^6 , or 10^9 colony-forming units (CFU) per ml. For the study of local infectivity, subcutaneous injections were made in the right groin with 0.2 ml of a bacterial suspension containing 10^9 CFU/ml. Two animals were used per strain. The mice were examined daily for swelling of lymph nodes. Evaluation and dissection of the mice followed after 7 days.

The local infectivity of anaerobic bacteria was studied by injecting 0.1 ml of a suspension (10^9 CFU/ml) of each *Rhodomicrobium* strain into a skin fold artificially made just behind the pivot joint in the back. This fold was made with a Michel clamp (7.5 by 1.75 mm; Aesculap, Tuttlingen, Federal Republic of Germany). The possible effects of the bacteria that caused organ changes in mice were also studied with guinea pigs injected intraperitoneally with 1-ml portions of washed suspensions (10^9 CFU/ml). In each experiment another guinea pig was injected with 1 ml of sterile PBS alone.

As positive controls, mice were injected with suspensions containing 5×10^7 CFU of 18-h cultures of pathogenic *Staphylococcus aureus* ATCC 6538 or *Escherichia coli* ATCC 11229 per ml. Additional control animals were injected with sterile PBS alone. All animals were examined several times daily for lethality, external infection symptoms, and any local changes or swellings for up to 21 days. Then the animals were sacrificed, dissected, and examined for possible internal changes. The organs (kidneys, livers, and spleens) were prepared aseptically for recultivation studies; they were rinsed and homogenized in sterile PBS, and 0.1-ml portions of the suspensions were inoculated onto suitable solid and liquid growth media. Organ suspensions of animals treated with rhodomicrobia were cultivated anaerobically.

Normalization of organs was also investigated. For this, strains which had previously caused spleen enlargement were reinoculated into six mice each, followed by weekly sacrifice of one mouse per strain from week 3 to week 8. These strains (namely, IFAM NQ-521gr, IFAM MEV-533gr, IFAM I-551, IFAM KB-677, and IFAM 1128) were also tested for toxicity. They were sonicated aseptically for 3 min in an ice bath (model W185F; Ultrasonics, Plainview, N.Y.), and 0.5 ml of each sonicated cell suspension was injected intravenously into a mouse (i.e., 5×10^8 CFU/ml). To determine the degree of disruption, 0.1 ml of each sonic extract was plated onto a suitable agar medium and cultivated.

Infectivity after immunosuppressive treatment. Strain C3H(H-2^b) mice were pretreated with the im-

munosuppressive drug Natulan [1-methyl-2-*p*-(isopropylcarbamoyl)benzylhydrazine hydrochloride; lot 0803001; LaRoche, Basel, Switzerland]. The doses used intravenously (and suggested by D. Ulrich, Universität Kiel, Federal Republic of Germany) were 0.5 ml of 1.39% solution per kg of mouse [i.e., 60% of a 50% lethal dose for mouse strain BALB/c(H-2^d)]. After 4 days the mice were infected with 0.5-ml portions of suspensions (10⁹ CFU/ml) of those strains which had caused changes of organs in prior experiments. Control animals, which were also pretreated with Natulan, were infected with 5 × 10⁷ CFU of *Staphylococcus aureus* or *Escherichia coli* per ml, as described above. Additional control animals were injected after Natulan treatment with sterile PBS alone.

Serological examinations (agglutination tests). Antibody determinations with the test strains were carried out from 7 to 21 days after inoculation. Blood from the cardiac area was kept at 22°C for 2 h and then at 4°C for 14 h. Serum was obtained by centrifuging the preparation twice at 5,000 × *g* for 5 min and then stored at -20°C until it was used. The yield of serum was about 50% of the blood volume collected. For qualitative antibody demonstrations we used the rocked slide method; 25 µl of serum was mixed with 25 µl of a cell suspension on precleaned, particle-free, glass slides, and the results were read after 3 to 5 min. Quantitative determinations were carried out with serial dilutions of sera in PBA by using microtiter plates (34) and young, homogenized, washed cell suspensions. Results were read after 14 h of incubation at 37°C by using a stereomicroscope at ×40 magnification. For cross-reaction experiments, equal volumes of an antiserum and a bacterial suspension (10⁹ CFU/ml) were mixed in microtiter plates and then treated as described above.

Histological studies. Immediately after dissection, organs were removed and fixed in 10% Formalin for 24 to 48 h at room temperature; this was followed by dehydration and paraffin embedding, using conventional techniques. Sections (5 µm) were cut with a microtome (model 1130; Jung AG, Heidelberg, Federal Republic of Germany) and were stained with periodic acid-Schiff reagent and hematoxylin or with hematoxylin and eosin.

The conventional Pappenheim staining procedure was used in part to demonstrate the possible presence of bacteria.

RESULTS

Pathogenicity. In an initial experiment, groups of three mice were injected intravenously in the tail with 1 of the 27 strains, using 0.5 ml of a washed cell suspension containing 10³, 10⁶, or 10⁹ CFU/ml. The subcutaneous challenges were done by using 0.2-ml portions of suspensions containing 10⁹ CFU/ml. All of the animals challenged by these techniques were alive after 7 days and survived inoculation without noticeable effects, such as local lesions, general abscesses, or microscopic changes. The four positive control mice, which were challenged with *Staphylococcus aureus* or *Escherichia coli*, died within 18 h after injection, whereas the negative

control mice injected with PBS alone survived without noticeable trauma.

Autopsies revealed that at challenge levels of 10⁹ CFU/ml four strains (IFAM NQ-521gr, IFAM MEV-533gr, IFAM I-551, and IFAM KB-677) of the *Hyphomicrobium* group and "Hyphomonas polymorpha" IFAM PR-727 induced enlargement of the spleens of mice. Strains IFAM 1128 and MEV-533gr caused slight enlargement and a mottled appearance of the kidney cortex. Strains of the genera *Prosthecomicrobium*, "Stella," *Rhodomicrobium*, *Pedomicrobium*, *Blastobacter*, *Planctomyces*, and *Pasteuria*, as well as the negative controls, did not cause any organ changes.

When mice were challenged with those strains that caused enlargement of the kidney cortex or spleen or both and subsequently sacrificed at a rate of one per week, no changes in the enlarged organs were observed for several weeks. Only with strain IFAM 1128 was there a slight reduction in spleen enlargement after 8 weeks.

Spleens, livers, or kidneys of mice infected with those strains which caused enlargement were examined for possible pathological changes. In addition, organs of apparently healthy mice which had been injected with other strains were also studied. Sections stained with periodic acid-Schiff reagent and hematoxylin or with hematoxylin and eosin showed no significant pathological changes, except perhaps a slight enlargement of spleen follicles and a dissemination of the splenic white pulp. Such changes were not seen in the PBS-treated control animals.

Survival of test bacteria in mice. Most bacteria could not be reisolated after 21 days from the spleens, livers, kidneys, or blood of the mice. Therefore, the survival times of those bacteria which caused spleen changes were tested by intravenous injection of 0.5-ml suspensions containing 10⁹ CFU/ml. Blood samples were taken daily for 10 days and streaked onto the appropriate agar media. Strain IFAM 1128 could not be reisolated after the first day, whereas *Hyphomicrobium* spp. strains IFAM NQ-521gr and IFAM KB-677 were still recovered after 2 days. Strains IFAM MEV-533gr and IFAM I-551 survived up to 4 days but with aberrant morphology.

In contrast to the other test strains, *Rhodomicrobium vanielii* IFAM 1093 and IFAM 1178^T survived for 10 days; these strains were readily reisolated from liver or spleen homogenates and were not aberrant (Fig. 1). Therefore, these two strains were injected (0.1 ml; 5 × 10⁸ CFU/ml) into clamped skin folds on mouse backs in an attempt to create more anaerobic conditions. However, neither local lesions nor abscesses developed, and there were no distinct organ changes in these animals. Blood samples con-

FIG. 1. Rhodomicrobium reisolated after 10 days from mouse organs. $\times 1,080$. (A) *R. vannielii* strain IFAM 1178^T reisolated from a liver suspension. (B) *R. vannielii* strain IFAM 1178^T reisolated from a spleen suspension. (C) *R. vaniellii* strain IFAM 1093 reisolated from a spleen suspension.

tained *Rhodomicrobium*-like structures, but these were about 10 times larger than the normal bacteria; these structures were not identified. Thus, it appears that the photosynthetic rhodomicrobium had a greater survival capacity in the hosts than the other bacteria tested.

Toxicity of sonicated cell suspensions. To determine the toxicity of disrupted cells, suspensions of strains IFAM NQ-521gr, IFAM MEV-533gr, IFAM I-551, IFAM KB-677, and IFAM 1128 were sonicated for 3 min, and 0.5 ml of each suspension was injected intravenously into an animal. Although cell breakage was not complete, the cells did not remain viable. None of

the test strains had the lethal endotoxic activity of *Escherichia coli*.

Antigenic properties of the budding and prosthecae bacteria. The serological agglutination reactions carried out on rocked slides showed qualitatively that all infected mice responded to antigens of the test bacteria. More quantitative tests in microtiter plates showed considerable variation in the agglutination titers; approximately one-half of the strains agglutinated at dilutions of 1:32 or 1:64. There was no correlation of titer with taxonomic position (Table 2).

Serological cross-reactions were studied by using mouse antisera and six bacterial strains,

TABLE 2. Agglutination reactions of bacterial strains with their homologous antisera

Strain(s)	Genus (known or suspected)	Highest dilution causing agglutination
IFAM 868, IFAM 869	<i>Pedomicrobium</i>	1:2
IFAM 1093	<i>Rhodomicrobium</i>	1:2
IFAM ZV-622	<i>Hyphomicrobium</i>	1:4
IFAM 1003	<i>Blastobacter</i>	1:4
IFAM 1009	<i>Pasteuria</i>	1:4
IFAM 1312	" <i>Stella</i> "	1:4
IFAM NQ-521gr, IFAM KB-677	<i>Hyphomicrobium</i>	1:8
IFAM PS-728	" <i>Hyphomonas</i> "	1:8
IFAM 1005	<i>Blastobacter</i>	1:8
IFAM H-526, IFAM CO-582	<i>Hyphomicrobium</i>	1:16
IFAM 1178 ^T	<i>Rhodomicrobium</i>	1:16
IFAM B-522, IFAM MEV-533gr, IFAM I-551	<i>Hyphomicrobium</i>	1:32
IFAM ZV-580, IFAM MC-748, IFAM WI-926	<i>Hyphomicrobium</i>	1:32
IFAM PR-727, IFAM 1325, IFAM LE-670	" <i>Hyphomonas</i> "	1:32
IFAM 1316	<i>Prosthecomicrobium</i>	1:32
IFAM 1007	<i>Planctomyces</i>	1:32
IFAM 1128	Unknown	1:32
IFAM MC-750	<i>Hyphomicrobium</i>	1:64

TABLE 3. Serological comparisons of several *Hyphomicrobium* strains isolated from soil and water

Taxon	Strain	Origin	Cross-reaction with antiserum against strain: ^a					
			IFAM B-522	IFAM I-551	IFAM NQ-521gr	IFAM MEV-533gr	IFAM KB-677	IFAM 1128
<i>Hyphomicrobium</i> spp.	IFAM B-522	Soil	+	+	—	—	—	—
	IFAM I-551	Soil	+	+	—	—	—	—
	IFAM NQ-521gr	Water	—	—	+	+	+	—
	IFAM MEV-533gr	Water	—	—	+	+	+	—
	IFAM KB-677	Sewage	—	—	+	+	+	—
Unknown	IFAM 1128	Water	—	—	—	—	—	+

^a +, Positive cross-reaction (agglutination of cells); —, negative cross-reaction.

five of which caused enlargement of spleens (*Hyphomicrobium* spp. strains IFAM NQ-521gr, IFAM MEV-533gr, IFAM I-551, and IFAM KB-677 and unnamed strain IFAM 1128). The sixth strain, *Hyphomicrobium* sp. strain IFAM B-522, has been widely used in studies of the genus *Hyphomicrobium* (9, 14–16, 24, 27). *Hyphomicrobium* spp. strains IFAM B-522 and IFAM I-551, which were isolated from soil and grew without pellicles, did cross-react with one another but not with any of the other three hyphomicrobia, which were isolated from brackish water or sewage and formed surface pellicles (Table 3). We observed no cross-reaction of strain IFAM 1128 with any of the other strains.

Infectivity after immunosuppressive treatment of the host. Brown female mice belonging to the pathogen-free strain C3H(H-2^k) were treated intravenously with 0.5 ml of a 1.39% Natulan solution per kg of mouse. After 4 days, these mice were challenged with suspensions containing (10⁹ CFU/ml) of those strains that induced enlargement of spleens, as described above. All animals, including the PBS-inoculated negative control, tolerated this immunosuppressive drug very well and survived inoculation. We observed neither symptoms of disease nor pathological changes of internal organs; bacteremia did not develop. However, four immunosuppressed mice challenged with the pathogenic strains of *Staphylococcus aureus* and *Escherichia coli* died within 8 h after infection.

Serological tests indicated that antibodies against the bacteria injected were not produced by the Natulan-treated hosts. However, all animals treated with Natulan, including the controls, had rather pale kidneys as a result of anemic reactions.

Biochemical activities as indicators of possible pathogenicity. Virulent bacteria, including *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Clostridium tetani*, have biochemical reactions that can be correlated with pathogenic-

ity (36). Therefore, the bacteria that caused enlargement of spleens or changes in kidneys were examined for hemolytic activities by inoculating sheep blood agar plates with young cell cultures. We observed no β -hemolysis with any strain; only “*Hyphomonas polymorpha*” strain IFAM PR-727 was α -hemolytic. None of the test strains catabolized mannitol.

In vivo pathogenicity tests with guinea pigs. Five guinea pigs were injected intravenously with 1-ml portions of PBS suspensions containing 10⁹ CFU of strains IFAM NQ-521gr, IFAM MEV-533gr, IFAM I-551, IFAM KB-677, IFAM PR-727, and IFAM 1128. After 14 days there were no obvious macroscopic symptoms of infection. When necropsied, the animals infected with strains IFAM NQ-521gr, IFAM MEV-533gr, and IFAM 1128 showed enlargement of the regional lymphatic nodes. Strain IFAM NQ-521gr induced some swelling of the paraaortic lymph nodes. This indicated the presence of effective defense mechanisms in the animal hosts.

Serological reactions carried out in microtiter plates by using antisera obtained from the infected animals showed host responses to the injected bacteria by antibody formation. Living bacteria could not be isolated from guinea pig organs.

DISCUSSION

Bacteria capable of causing disease can be widely distributed in natural environments and escape detection, as seen from the recent discovery of *Legionella* spp. (3). It was for this reason that 27 bacteria that are budding or prosthecate or both were tested for possible pathogenicity in the most extensive study to date (31, 32; R. Gebers, Ph.D. thesis, Universität Kiel, Kiel, Federal Republic of Germany, 1978).

The results obtained with the *Hyphomicrobium* strains tested are consistent with the ob-

servation that these organisms are oligotrophic utilizers of C-1 compounds (13, 14) and are inhibited in the presence of low concentrations of certain amino acids (N. Matzen, personal communication). Thus, we can assume, at least for these bacteria, that the nutritional environment in the host is inadequate (10, 35). In most cases the maximum temperature for growth of the test strains was above 37°C.

In our studies pathogenicity was tested in different ways. In addition to the tests for the ability to utilize mannitol and the ability to cause β -hemolysis, we performed in vivo tests in which we used several routes of infection and different bacterial concentrations. Also, tests for serological reactions and infection after suppression of the host immunological defense system were performed. None of these tests indicated pathogenicity. Animals are considered appropriate to indicate the pathogenic potential of bacteria for humans; in our studies guinea pigs were used in addition to mice, since host susceptibility is regarded as specific generic property of animals (38).

The actual cell densities injected were calculated from measurements of optical density at 650 nm, since parallel determinations of viable counts showed that there was a good correlation between the optical density at 650 nm and the number of CFU per milliliter. However, several of the test strains (*Hyphomicrobium*, *Blastobacter*, and *Pasteuria* strains) tended to clump or even form rosettes, and in these cases the actual numbers injected would have been higher than the numbers obtained from plate counts.

Initially, the concentrations of bacteria injected were varied from 10^3 to 10^9 CFU/ml. When we found that even the highest cell density did not seem to affect the hosts, experiments were carried out with this concentration.

The bacteria were administered by different routes. Intravenous injection was used to show the degree of pathogenicity (i.e., virulence) and to determine the bacterial doses necessary to cause disease. Subcutaneous injections were made to study local infectivity, and injections into clamped skin folds were made to create an environment in the hosts as anaerobic as possible to suit the normally anaerobic *Rhodomicrobium* strains. Interestingly enough, *Rhodomicrobium* strains were the only bacteria that could be reisolated from the animals after 10 days. A possible explanation for this is that the *Rhodomicrobium* suspensions may have contained cysts, a type of resting and heat-resistant cell with a certain resemblance to spores (12). The rapid disappearance of the other test bacteria from the host bloodstreams could have been caused by (i) nutritional unsuitability of the host environment or (ii) the presence of an effective

defense system in the host. Resistance could be related to an active reticuloendothelial system, which is known to cause phagocytosis and destruction of bacteria circulating in the blood. The presence of antibacterial substances in the bloodstream or serum of a host could also account for the inability of the bacteria to survive (7, 28).

The in vivo tests with mice and guinea pigs revealed neither virulence nor toxicity in the 27 bacteria. Enlargement of spleens and a mottled appearance of kidneys in the case of some closely related strains were evidently normal host reactions to the presence of the bacteria. A histological examination of the enlarged spleens revealed minor follicular changes and dissemination of the splenic white pulp, which can be attributed to increased stimulation of cellular defense (6, 33, 34), to the elimination process of the spleen (5), or to antibody-antigen complex reactions (26).

The agglutination tests with antisera and the corresponding strains showed that all animals responded to the presence of the bacteria by antibody formation. Therefore, serological cross-reactions could be carried out with selected strains to look for antigenic relationships. The few preliminary experiments which we performed indicated that *Hyphomicrobium* strains obtained from soil cross-reacted, but that these strains did not react with aquatic strains (Table 3). This demonstrates the presence in the genus *Hyphomicrobium* of different taxonomic subgroups, an observation made previously by Mandel et al. (24), Moore and Hirsch (27), and Powell et al. (32).

The non-infectivity of the 27 strains could be demonstrated after immunosuppressive treatment of mice. Natulan, a substance used in transplantation work, affects mainly the humoral response of a host (2), yet the infectious capacities of our test bacteria were not enhanced. Lack of virulence in our strains could have been due to continued culture on laboratory media. To test this, new isolates would have had to be isolated and maintained on media promoting virulence. The low virulence of this group of bacteria was underscored by the inability of 10 strains to utilize mannitol or cause β -hemolysis (36).

ACKNOWLEDGMENTS

We acknowledge receipt of bacterial cultures from W. Mevius, Jr., G. A. Zavarzin, J. Y. Kingma-Boltjes, E. Leifson, E. Pongratz, W. Babinchak, and F. Eckhardt. We also appreciate the help in the histological parts of this work kindly rendered by our colleagues L. Rath, E. Haase, and H. K. Müller-Hermelink (Universität Kiel).

LITERATURE CITED

1. Austin, B., D. A. Allen, A. Zachary, M. R. Belas, and R. R. Colwell. 1979. Ecology and taxonomy of bacteria

- attaching to wood surfaces in a tropical harbour. Can. J. Microbiol. **25**:447-461.
2. **Bach, J. F.** 1975. The mode of action of immunosuppressive agents. North-Holland Publishing Co., Amsterdam.
 3. **Brenner, D. J., A. G. Steigerwalt, and J. E. McDade.** 1979. Classification of the Legionnaire's disease bacterium: *Legionella pneumophila*, genus novum, species nova, of the family *Legionellaceae*, familia nova. Ann. Intern. Med. **90**:656-658.
 4. **Buchanan, R. E., and N. E. Gibbons (ed.).** 1974. Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
 5. **Burnett, F. M.** 1962. Natural history of infectious disease. Cambridge University Press, London.
 6. **Congdon, C. C., and T. Makinodan.** 1961. Splenic white pulp alteration after injection: relation to time of serum antibody production. Am. J. Pathol. **39**:697-709.
 7. **Cybulska, J., and J. Jeljaszewicz.** 1966. Bacteriostatic activity of serum against *Staphylococcus*. J. Bacteriol. **91**:953-962.
 8. **Duchow, E., and H. C. Douglas.** 1949. *Rhodomicrobium vannielii*, a new photoheterotrophic bacterium. J. Bacteriol. **91**:409-416.
 9. **Eckhardt, F., P. Roggentin, and P. Hirsch.** 1979. Fatty acid composition of various hyphal budding bacteria. Arch. Microbiol. **120**:81-85.
 10. **Garber, E. D.** 1960. The host as a growth medium. Ann. N.Y. Acad. Sci. **85**:1187-1194.
 11. **Ghiorse, W. C., and P. Hirsch.** 1979. An ultrastructural study of iron and manganese deposition associated with extra-cellular polymers of *Pedomicrobium*-like bacteria. Arch. Microbiol. **123**:213-226.
 12. **Gorlenko, V. M.** 1969. Spore formation in a budding photoheterotrophic bacterium. Mikrobiologiya **38**:106-111.
 13. **Harder, W., and M. M. Attwood.** 1978. Biology, physiology and biochemistry of *Hyphomicrobium*. Adv. Microb. Physiol. **17**:303-359.
 14. **Hirsch, P.** 1974. Budding bacteria. Annu. Rev. Microbiol. **28**:391-444.
 15. **Hirsch, P., and S. F. Conti.** 1964. Biology of budding bacteria. I. Enrichment, isolation and morphology of *Hyphomicrobium* spp. Arch. Mikrobiol. **48**:339-357.
 16. **Hirsch, P., and S. F. Conti.** 1964. Biology of budding bacteria. II. Growth and nutrition of *Hyphomicrobium* spp. Arch. Mikrobiol. **48**:358-367.
 17. **Hirsch, P., M. Müller, and H. Schlesner.** 1977. New aquatic budding and prosthecae bacteria and their taxonomic position. Soc. Appl. Bacteriol. Symp. Ser. **6**:107-133.
 18. **Hirsch, P., and G. Rheinheimer.** 1968. Biology of budding bacteria. V. Budding bacteria in aquatic habitats: occurrence, enrichment and isolation. Arch. Mikrobiol. **62**:289-306.
 19. **Hurlbert, E., J. Weckesser, H. Mayer, and I. Fromme.** 1976. Isolation and characterization of the lipopolysaccharide of *Chromatium vinosum*. Eur. J. Biochem. **68**:365-371.
 20. **Katz, A., J. Weckesser, and G. Drews.** 1977. Chemical and biochemical studies on the lipopolysaccharide (O-antigen) of *Anacystis nidulans*. Arch. Microbiol. **113**:247-256.
 21. **Kingma-Boltjes, J. Y.** 1936. Über *Hyphomicrobium vulgare* Stutzer & Hartleb. Arch. Mikrobiol. **7**:188-205.
 22. **Leifson, E.** 1964. *Hyphomicrobium neptunium* sp.n. Antonie van Leeuwenhoek J. Microbiol. Serol. **30**:249-256.
 23. **Lyman, J., and R. H. Fleming.** 1940. Composition of seawater. J. Mar. Res. **3**:134-146.
 24. **Mandel, M., P. Hirsch, and S. F. Conti.** 1972. Deoxyribonucleic acid base compositions of hyphomicrobia. Arch. Mikrobiol. **81**:289-294.
 25. **Mevius, W., Jr.** 1953. Beiträge zur Kenntnis von *Hyphomicrobium vulgare* Stutzer & Hartleb. Arch. Mikrobiol. **19**:1-29.
 26. **Miescher, P. A., and H. J. Müller-Eberhard.** 1968. Textbook of immunopathology, vol. 1. Grune & Stratton, New York.
 27. **Moore, R. L., and P. Hirsch.** 1972. DNA base sequence homologies of some budding and prosthecae bacteria. J. Bacteriol. **110**:256-261.
 28. **Muschel, L. H.** 1960. Serum bactericidal action. Ann. N. Y. Acad. Sci. **88**:1265-1272.
 29. **Pfennig, N.** 1969. *Rhodopseudomonas acidiphila* sp.n., a new species of the budding purple nonsulfur bacteria. J. Bacteriol. **99**:597-602.
 30. **Pfennig, N., and K. D. Lippert.** 1966. Über das Vitamin B₁₂-Bedürfnis phototropher Schwefelbakterien. Arch. Mikrobiol. **55**:258-266.
 31. **Pongratz, E.** 1957. D'une bactérie pédiculée isolée d'un pus de sinus. Schweiz. Z. Pathol. Bakteriologie **20**:593-608.
 32. **Powell, D. M., B. S. Roberson, and R. M. Weiner.** 1980. Serological relationships among budding, prosthecae bacteria. Can. J. Microbiol. **26**:209-217.
 33. **Robbins, S. L.** 1974. Pathologic basis of disease. Saunders Co., Philadelphia.
 34. **Roitt, I.** 1977. Essential immunology, 3rd ed. Blackwell Scientific Publications, London.
 35. **Smith, H.** 1976. Survival of vegetative bacteria in animals. Symp. Soc. Gen. Microbiol. **26**:299-326.
 36. **Sonntag, H. G., and K. Nachtigall.** 1977. Vergleichende Untersuchungen über biochemische Pathogenitätsmerkmale und Resistenzverhalten gegen Antibiotika bei Staphylokokken. Zentralbl. Bakteriologie. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **238**:310-319.
 37. **Staley, J. T.** 1968. *Prosthecomicrobium* and *Ancalomicrobium*: new prosthecae freshwater bacteria. J. Bacteriol. **95**:1921-1942.
 38. **Strong, L. C.** 1978. Origins of inbred mice. Academic Press, Inc., New York.
 39. **Van Ert, M., and J. T. Staley.** 1971. Gas-vacuolated strains of *Microcyclops aquaticus*. J. Bacteriol. **108**:236-240.
 40. **Vasil'eva, L. V.** 1970. A star-shaped soil microorganism. Izv. Akad. Nauk SSSR Ser. Biol. **2**:308-310.
 41. **Weckesser, J., G. Drews, and H. Mayer.** 1977. Lipopolysaccharide aus Zellwänden photosynthetischer Prokaryonten: Bedeutung für Fragen der Taxonomie und der endotoxischen Aktivität. Naturwiss. Rundsch. **30**:360-364.
 42. **Zavarzin, G. A.** 1960. The life-cycle and nuclear apparatus in *Hyphomicrobium vulgare* Stutzer and Hartleb. Mikrobiologiya **29**:24-27.