# CHARACTERIZATION OF *PEPTOSTREPTOCOCCUS* SPECIES BY AMINOPEPTIDASE PROFILES AND GENOTYPING METHODS.

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## ABSTRACT

*Peptostreptococcus* species are anaerobic Gram-positive cocci associated with a variety of female genital tract infections that includes bacterial vaginosis and pelvic inflammatory disease. Their role in such infections is not well characterized partly because of difficulties in isolation and they are often not classified. Also, their classification is currently undergoing taxonomic changes and there is a need to identify virulence factors, and a requirement to determine the genetic relationship among pathogenic strains. One of the accepted indicators in bacterial vaginosis is the production of proteases which involves *Peptostreptococcus* spp. One hundred *Peptostreptococcus* isolates were screened for aminopeptidase activities using the biochemical kit, rapid ID32A (bioMérieux Vitek, Inc., Hazelwood, MI ) and for proteolytic activity using gelatin hydrolysis. Five ATCC *Peptostreptococcus* strains and 95 clinical isolates comprising *P. anaerobius*, *P. asaccharolyticus*, *P. magnus*, *P. micros*, and *P. prevotii* were included. Most *P. magnus* (23/24; 95.8%) and *P. micros* isolates (15/19; 79%) hydrolyzed gelatin whereas few *P. asaccharolyticus* (2/25; 8%), *P. anaerobius* (2/20; 10%), and *P. prevotii* isolates (2/12; 16.7%) were able to do so. Statistical analysis showed that the prevalence of gelatin hydrolysis is significantly correlated to the number of positive aminopeptidase reactions to synthetic peptides among the 5 *Peptostreptococcus* species ($r$=0.80; $P$=0.0019). *P. magnus* and *P. micros* isolates had the greatest array of aminopeptidase reactions and reflected the higher percentage of these isolates that hydrolyzed gelatin. Semi-quantitative assessment of proteolytic activity for 4 *P. magnus* isolates was weak compared to the highly proteolytic organism *Porphyromonas gingivalis* W83 when assayed on general and nonspecific substrates such as azocoll, azocasein, benzoyl-arginine-*p*-nitroanilide, bovine serum albumin, casein, casein yellow, collagen, autoclaved collagen,

fibrinogen, gelatin, and glycyl-L-proline-*p*-nitroanilide. Therefore, proteolytic activity of *Peptostreptococcus* species, in particular *P. magnus* and *P. micros*, is due in part to the array of aminopeptidase reactions detected.

The rapid ID32A kit was also used to identify the 100 *Peptostreptococcus* isolates as compared to conventional biochemical tests and gas-liquid chromatography, the current "gold" standard method. Also, the kit was evaluated for its sensitivity and specificity. Overall, the sensitivity and specificity of the rapid ID32A kit in the identification for 5 *Peptostreptococcus* species as compared to the standard method was 93% and 80% respectively. All *P. anaerobius* ($n$=20) and *P. asaccharolyticus* ($n$=25) isolates were identified with 100% sensitivity and 100% specificity. For the identification of *P. magnus* ($n$=24) and *P. micros* ($n$=19), the rapid ID32A kit was 100% sensitive for both species while the specificity for *P. magnus* was 95.8% and 57.9% for *P. micros*. The lower specificity for *P. micros* was due to eight of 11 female genital tract isolates being identified as *P. magnus*. The sensitivity and specificity of the rapid ID32A kit for identification of *P. prevotii* ($n$=12) was poor (41.7% and 8.3%, respectively) and may be due to the initial incapability of the "gold" standard method to differentiate these clinical isolates. The rapid ID32A kit is a useful method for the rapid differentiation of *P. anaerobius* and *P. asaccharolyticus* from other *Peptostreptococcus* spp. Alternate methods should be used to speciate isolates of *P. magnus*, *P. micros*, and *P. prevotii*.

Protein L (L) and albumin-binding (A) proteins have been identified as putative virulence factors of *P. magnus* isolates. When 32 *P. magnus* isolates from 4 different countries were screened for protein L gene sequences by the polymerase chain reaction (PCR), the only positive isolates were three which had been previously identified. Albumin-binding isolates were recovered from both Sweden and Canada. In order to determine whether these putative virulence factors might be clonal,

protein L-containing ($L^+ A^-$) or albumin-binding isolates ($L^- A^+$) and unrelated isolates ($L^- A^-$) were analyzed using three genotyping methods- PCR-restriction endonuclease analysis (REA) of 16S rRNA, ribotyping, and macro restriction endonuclease analysis of DNA by pulsed-field gel electrophoresis (PFGE). PFGE produced the greatest discrimination (ten banding patterns), followed by ribotyping (nine banding patterns) and PCR-REA of 16S rRNA amplicons (two banding patterns). Based on patterns from PFGE and ribotyping, cluster analysis showed that the three $L^+A^-$ isolates were more closely related to each other than with other isolates. Six of eight $L^- A^+$ isolates formed two indistinguishable groups of three isolates each, but these two groups were genetically distant from the other two unrelated $L^- A^+$ isolates. $L^- A^-$ isolates were not related, either among themselves, or to any other isolates. Thus, $L^+$ and $A^+$ phenotypes and genotyping methods (PFGE and ribotyping) are useful for differentiating *P. magnus* isolates and identifying specific strain types.

In conclusion, my studies have found that the proteolytic activity of *Peptostreptococcus* spp. is actually due in part to an array of aminopeptidase reactions. As well, certain isolates of *Peptostreptococcus* species require further taxonomic work which will likely lead to changes in classification and nomenclature. All 11 clinical isolates originally identified as *P. prevotii* by the "gold" standard methodology were either identified as *P. magnus* ($n$=4) or could not be identified by the rapid ID32A kit. Protein L-containing and some albumin-binding *P. magnus* isolates are clonal in structure. This is in agreement with past studies that most pathogenic isolates of bacteria are descendent from one parental strain.

# RÉSUMÉ

Les espèces de *Peptostreptococcus* sont des coques Gram-positifs associés à diverses infections de l'appareil génital féminin dont la vaginose bactérienne et la maladie pelvienne inflammatoire. Leur rôle dans ces infections n'est pas bien caractérisé en partie à cause des difficultés associées à leur isolation et souvent ils ne pas classés. De plus, leur classification subit en ce moment des changements taxonomiques et il y existe un besoin d'identifier des facteurs de virulence ainsi qu'un besoin de déterminer le lien génétique entre les souches pathogènes. Un des indicateurs reconnus de la vaginose bactérienne est la production de protéases, ce qui implique la présence d'espèces de *Peptostreptococcus*. Cent isolats de *Peptostreptococcus* furent examinés pour la présence d'activités aminopeptidase en utilisant la trousse biochimique rapid ID32A (bioMérieux Vitek, Inc., Hazelwood, MI.) ainsi que pour la présence d'activité protéolytique en utilisant l'hydrolyse de la gélatine. Cinq souches ATCC de *Peptostreptococcus* et 95 isolats cliniques incluant *P. anaerobius*, *P. asaccharolyticus*, *P. magnus*, *P. micros* et *P. prevotii* furent inclus. La majorité des isolats de *P. magnus* (23/24; 95.8%) et de *P. micros* (15/19; 79%) hydrolysaient la gélatine, tandis que seulement quelques isolats de *P. asaccharolyticus* (2/25; 8%), de *P. anaerobius* (2/20; 10%) et de *P. prevotii* (2/12; 16.7%) en avaient la capacité. L'analyse statistique a démontré que la prévalence de l'hydrolyse de la gélatine est significativement liée au nombre de réactions aminopeptidase positives à des peptides synthétiques présentes dans le profil des isolats de *Peptostreptococcus* ($r$=0.80; $P$=0.0019). Les isolats de *P. magnus* et de *P. micros* avaient la plus grande ensemble de réactions aminopeptidase et ceci réflétait le pourcentage plus élevé de ces isolats qui hydrolysaient la gélatine. L'évaluation semi-

quantitative de l'activité protéolytique pour 4 isolats de *P.magnus* était faible comparativement à celle de *Porphyromonas gingivalis* W83, un organisme possédant une activité protéolytique élevée, lorsque testée avec des substrats généraux et non-spécifiques tels que l'azocoll, l'azocaséine, le benzoyl-arginine-*p*-nitroanilide, l'albumine de sérum bovin, la caséine, la caséine jaune, le collagène, le collagène autoclavé, la fibrinogène, la gélatine et le glycyl-L-proline-*p*-nitroanilide. Ainsi, l'activité protéolytique des espèces de *Peptostreptococcus*, en particulier *P. magnus* et *P. micros*, est due en partie au grand ensemble de réactions aminopeptidase détectées.

La trousse rapid ID32A fut également utilisé pour identifier les mêmes 100 isolats de *Peptostreptococcus* comparativement à des tests biochimiques conventionnels et à la chromatographie gazeuse-liquide, la méthode de référence standard. La sensibilité et la spécificité de ce kit furent aussi évaluées. En tout et partout, la sensibilité et la spécificité du kit rapid ID32A furent respectivement 93% et 80% pour l'identification de 5 espèces de *Peptostreptococcus* comparativement à la méthode standard. Tous les isolats de *P. anaerobius* ($n$=20) et de *P. asaccharolyticus* ($n$=25) furent identifiés avec 100% de sensibilité et 100% de spécificité. Quant à l'identification de *P. magnus* ($n$=24) et de *P. micros* ($n$=19), la sensibilité de la trousse rapid ID32A fut de 100% pour les deux espèces tandis que la spécificité fut de 95.8% pour *P. magnus* et de 57.9% pour *P. micros*. La spécificité plus faible pour *P. micros* fut attribuée au fait que huit de 11 isolats provenant de l'appareil génital féminin furent identifiés comme étant *P. magnus*. La sensibilité et la spécificité de la trousse rapid ID32A pour l'identification de *P. prevotii* ($n$=12) étaient faibles (respectivement 41.7% et 8.3%) et pourraient être dues à l'incapacité initiale de la méthode de référence standard à différencier ces isolats cliniques. La

trousse rapid ID32A est une méthode utile pour la différenciation rapide de *P. anaerobius* et de *P. asaccharolyticus* des autres espèces de *Peptostreptococcus*. D'autres méthodes devraient être utilisées pour spécifier les isolats de *P. magnus*, *P. micros* et *P. prevotii*.

La protéine L (L) et la protéine liant l'albumine (A) ont été identifiées comme des facteurs putatifs de virulence chez des isolats de *P. magnus*. Lorsque 32 isolats de *P. magnus* provenant de quatre pays furent inspectés pour la présence des séquences de gènes codant pour la protéine L par la réaction en chaîne de la polymérase (PCR), les seuls isolats positifs furent les trois qui avaient été précédemment identifiés. Les isolats liant l'albumine furent identifiés parmi des échantillons provenant de la Suède et du Canada. Afin de déterminer si ces facteurs putatifs de virulence pourraient avoir une structure clonale, des isolats contenant la protéine L ($L^+A^-$) ou contenant la protéine liant l'albumine ($L^-A^+$) et d'autres isolats sans relation avec les deux premiers groupes ($L^-A^-$) furent analysés par trois méthodes de génotypage- la PCR-analyse par endonucléases de restriction (REA) de l'ARNr 16S, le ribotypage, et l'analyse des fragments de restriction de l'ADN par électrophorèse en champ pulsé (PFGE). Le PFGE démontre le plus grand pouvoir discriminant (dix patrons de bandes), suivi du ribotypage (neuf patrons de bandes) et du PCR-REA des amplicons de l'ARNr 16S (deux patrons de bandes). A partir des patrons du PFGE et du ribotypage, l'analyse des groupes a montré que les trois des isolats $L^+A^-$ étaient davantage liés entre eux qu'avec les autres isolats. Six des huit isolats $L^-A^+$ formaient deux groupes indifférenciables contenant chacun trois isolats, mais ces deux groupes étaient génétiquement éloignés des deux autres isolats $L^-A^+$. Les isolats $L^-A^-$ n'étaient pas parents, pas plus entre eux qu'avec tout autre isolat. Ainsi, les phénotypes $L^+$ et $A^+$ et les méthodes de génotypage (PFGE et

ribotypage) sont utiles pour différencier les isolats de *P. magnus* et pour identifier les types de souches spécifiques.

En conclusion, mes travaux ont montré que l'activité protéolytique des espèces de *Peptostreptococcus* est en fait partiellement due à un ensemble de réactions aminopeptidase. De plus, davantage de caractérisation taxonomique est requise dans le cas de certains isolats de *Peptostreptococcus*, ce qui mènera à des changements de classification et de nomenclature. Les 11 isolats cliniques originalement identifiés soit comme étant *P. prevotii* par la méthode de référence standard furent identifiés comme étant *P. magnus* ($n$=4) ou ne purent être identifiés avec le kit rapid ID32A. Les isolats de *P. magnus* contenant la protéine L et certains isolats contenant la protéine liant l'albumine ont une structure clonale. Ceci est en accord avec des études antérieures ayant démontré que la plupart des isolats pathogènes de bactéries sont issues d'une souche parentale.

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## ABBREVIATIONS

**A :** Albumin-binding

**aa:** Amino acid

**ADH:** Arginine dihydrolase

**ALA:** Alanine arylamidase

**Ap:** Ampicillin

**αARA:** Alpha arabinosidase

**ARG:** Arginine dihydrolase

**ATCC:** American Type Culture Collection

**BAPNA:** Benzoyl-arginine-*p*-nitroanilide

**BHI:** Brain heart infusion

**BSA:** Bovine serum albumin

**BV:** Bacterial vaginosis

**CAM:** Chorioamniotic membrane

**CBA:** Columbia blood agar

**CIAP:** Calf intestinal alkaline phosphatase

**dd$H_2O$:** Double distilled water

**DIG:** Digoxigenin

**DNA:** Deoxyribonucleic acid

**EDTA:** Disodium ethylenediaminetetraacetate·d$H_2O$

**ERIC:** Enterobacterial repetitive intergenic consensus

**αFUC:** Alpha fucosidase

**αGAL:** Alpha galactosidase

**βGAL:** Beta galactosidase

**GDC:** Glutamic acid decarboxylase

**GG:** Glutamyl glutamic acid arylamidase

**GLC:** Gas-liquid chromatography

**αGLU:** Alpha glucosidase

**βGLU:** Beta glucosidase

**GLY:** Glycine arylamidase

**Gly-Pro-Na:** Glycine-L-proline-*p*-nitroanilide

**βGP:** Beta galactosidase 6 phosphate

**GPAC:** Gram positive anaerobic coccus

**βGUR:** Beta glucurunidase

**HCl:** Hydrochloric acid

**HIS:** Histidine arylamidase

**Ig:** Immunoglobulin

**IND:** Indole production

**IUD:** Intrauterine device

**Km:** Kanamycin

**L:** Protein L

**LB:** Luria broth

**LEU:** Leucine arylamidase

**LG:** Leucyl glycine arylamidase

**Macro REA:** macro restriction endonuclease analysis

**Micro REA:** micro restriction endonuclease analysis

**MLEE:** Multilocus enzyme electrophoresis

**MNE:** Mannose fermentation

**βNAG:** Beta N-acetyl-glucosaminidase

**NBT:** Nitroblue tetrazolium chloride

**NIT:** Nitrate reduction

**NZY:** NZ amine: casein hydrolysate enzymatic

**OD:** Optical density

**OTU:** Operational taxonomic unit

**PAB:** Peptostreptococcal albumin-binding

**PAL:** Alkaline phosphatase

**PBS:** Phosphate-buffered saline

**PCR:** Polymerase chain reaction

**PEG:** Polyethylene glycol

**PFGE:** Pulsed-field gel electrophoresis

**PHE:** Phenylalanine arylamidase

**PID:** Pelvic inflammatory disease

**PMNLs:** Polymorphonuclear leucocytes

**PPL:** Peptostreptococcal protein L

**PRO:** Proline arylamidase

**PROM:** Premature rupture of membrane

**PY:** Peptone yeast extract broth

**PYR:** Pyroglutamic acid arylamidase

**RAF:** Raffinose fermentation

**REA:** Restriction endonuclease analysis

**REP:** Repetitive extragenic palindrome

**RFLPs:** Restriction fragment length polymorphisms

**SDS:** Sodium dodecyl sulphate

**SER:** Serine arylamidase

**STD:** Sexually transmitted disease

**TCA:** Trichloroacetic acid

**TLC:** Thin-layer chromatography

**TOAs:** Tubo-ovarian abscesses

**TSA** - Trypticase Soya agar

**TYR:** Tyrosine arylamidase

**UPGMA:** Unweighted pair-group method arithmetic averages

**URE:** Urease

**VPI:** Virginia Polytechnic Institute

**X-GAL:** 5-bromo-4-chloro-3-indolyl-β-D-galactoside

**X-Phosphate** - 5-bromo-4-chloro-3-indolylphosphate

# 1. INTRODUCTION

## 1.1 INVOLVEMENT OF *PEPTOSTREPTOCOCCUS* SPECIES IN FEMALE GENITAL TRACT INFECTIONS.

The most common endogenous anaerobes isolated from female genital tract infections, besides Gram-negative bacilli (*Bacteroides* spp., *Prevotella* spp., and *Porphyromonas* spp.), are the Gram-positive cocci *Peptostreptococcus* spp. (35, 72, 83, 198). Most female genital tract infections causing bacterial vaginosis (BV), premature rupture of membranes (PROM), intraamniotic infections, chorioamnionitis, postpartum endometritis, vaginal-cuff cellulitis, and pelvic inflammatory disease (PID) are polymicrobial involving aerobes, facultative anaerobes, and anaerobes (48, 64, 83, 173). Such mixed infections are often synergistic in nature (33-34, 36-38). As well, *Peptostreptococcus* spp. have been isolated in pure culture from a variety of bone or joint infections and soft tissue infections (28, 55, 174, 210, 217, 224, 241, 303). Historically, the pathogenic potential of *Peptostreptococcus* spp. in female genital tract infections has been overlooked for several reasons that include difficulties in identification (73, 102, 180, 186), an evolving taxonomy (70, 111, 142-143, 180, 309), and an undefined role partly due to their low virulence (28, 33, 38, 83, 198). Common *Peptostreptococcus* species associated with female genital tract infections include *P. anaerobius*, *P. asaccharolyticus*, *P. magnus*, *P. micros*, *P. prevotii*, *P. vaginalis*, and *P. tetradius* (53, 83, 101, 198). *P. vaginalis* has been isolated only from humans (142).

## 1.2 ROLE OF *PEPTOSTREPTOCOCCUS* SPECIES IN BACTERIAL VAGINOSIS.

BV is one of the most common vaginal infections and is characterized by an increased vaginal discharge without inflammation (98, 107). Bacteriologically, BV is characterized by an overgrowth

of anaerobes in a synergistic relationship with *Gardnerella vaginalis*, *Prevotella* spp., *Porphyromonas* spp., *Mobiluncus* spp., *Propionibacterium* spp., *Mycoplasma hominis*, and *Peptostreptococcus* spp. with a concomitant decrease of $H_2O_2$-generating *Lactobacillus* spp. (100, 123, 216, 261, 285). *G. vaginalis*, *M. hominis*, *Mobiluncus mulieris*, and *Mobiluncus curtisii* are associated with BV and are also located in the intestinal tract suggesting a mode of transmission to the vagina but the mechanism was not specified (108).

The current standard for the diagnosis of BV consists of identifying the presence of clue cells (at least 20% of vaginal epithelial cells coated with *G. vaginalis* and/or *Mobiluncus* spp.) on a wet mount and two of the three following indicators: 1) an elevated vaginal fluid pH > 4.5; 2) a thin, white, homogeneous vaginal discharge and; 3) a fishy amine odour in the vaginal fluid after the addition of 10% KOH (149, 163). Other previous tests include Gram stains of vaginal smears, which have the advantage of being inexpensive, quick, widely available, and have intra- and intercenter reliability (161, 205), the culture of *G. vaginalis* (84), the detection of volatile fatty acids (218, 262) and amines/diamines in the vaginal fluid by gas-liquid chromatography (GLC) or thin-layer chromatography (TLC) (30, 44-45), and increased levels of proline aminopeptidase in the vaginal fluid (284).

Although the exact role of *Peptostreptococcus* species in BV is presently unknown, there is an association of certain *Peptostreptococcus* species with BV (101). *Peptostreptococcus* species common to BV include *P. asaccharolyticus*, *P. anaerobius*, *P. magnus*, *P. prevotii*, and *P. tetradius* (97). Based on the bacteriological characteristics of BV, *P. anaerobius* and *P. tetradius* have been shown to be positively associated with BV and with reduced numbers of $H_2O_2$-generating lactobacilli and are considered as part of the BV complex (101). *P. magnus* and *P. asaccharolyticus* have been

isolated at similar frequencies in the presence or absence of $H_2O_2$-generating lactobacilli and are not considered as part of the BV complex (101). *P. prevotii* is positively associated with BV but appears not to be inhibited by $H_2O_2$-generating lactobacilli (101). Only one putative virulence factor has been found that has been correlated with *Peptostreptococcus* species and BV. Protein L ($PPL_{312}$) (peptostreptococcal protein L) is an Ig-binding protein (116). Protein L was found on < 10% (4/30) of isolates of *P. magnus* whereas > 50% (4/7) of BV strains contained protein L isolated from patients with BV (116). Statistical analysis showed that there was a correlation between protein L expressing *P. magnus* isolates and BV (116). $PPL_{312}$ was sequenced from one isolate (117).

In the vaginal fluid of patients with BV, there is an increase in succinate, butyrate, acetate, and propionate whereas lactate is reduced (261). A succinate:lactate ratio of ≥ 0.4 is diagnostic of BV with the rise in succinate due to increased numbers of *Prevotella* spp. and *Mobiluncus* spp. and the reduction in lactate due to the fall in lactobacilli (61, 260). Part of the increase in acetate and butyrate is due to *Peptostreptococcus* species (20, 260-261). *In vitro* studies have shown that succinic acid at concentrations up to 31 mM and at pH 5.5 (conditions found in abscesses and BV) markedly impair the phagocytic killing capacity of polymorphonuclear leukocytes and reduced the ability of these cells to respond to chemotactic stimuli (233).

The proteolytic degradation of collagen in tissue framework structures within the female genital tract may leave proline containing peptides which may serve as substrates and could account for the elevated levels of proline aminopeptidase seen in the vaginal fluids of BV patients (248, 250). Proline aminopeptidase is likely to be the product of the anaerobic flora (248). The anaerobic flora is increased during BV and many of these organisms produce proline aminopeptidase (248, 284). *P. anaerobius* is one such organism that produces proline aminopeptidase (69, 182, 184), is positively

associated with the BV complex (101), and likely a contributor to the increased levels. Under anaerobic conditions, the production of proline aminopeptidase benefits the growth of *P. anaerobius* since proline serves as an electron acceptor which allows for the uptake of glucose and alanine as energy sources (54).

Metronidazole is usually effective in treating BV (171). However, one study showed that metronidazole reduced most of the symptoms associated with BV, but not all, by eliminating the anaerobic flora except for *Peptostreptococcus* (171). This indirectly suggests that *Peptostreptococcus* species play a role in BV and some of the peptostreptococci are resistant to metronidazole.

**1.3 PREGNANCY COMPLICATIONS.**

BV and BV-associated bacteria have been shown to increase the risk factor of certain complications during pregnancy that include amniotic infection, preterm delivery, PROM, premature labor, and chorioamnionitis (64). The most likely pathway for these bacteria is via an ascending route (232). As part of the BV complex, *Peptostreptococcus* spp. have been isolated from all these upper genital tract infections associated with pregnancy. *P. anaerobius*, *P. asaccharolyticus* and *P. magnus* were shown to be significantly associated with preterm delivery, chorioamnion infections, and/or chorioamnionitis (32, 99, 109, 268). In another study, *Peptostreptococcus* spp. along with enteropharyngeal bacteria were acquired in the late stages of pregnancy with preterm births and their persistence from midtrimester was rare (164). Amniotic fluid has antibacterial properties such as lysozyme, B-lysin, transferrin, immunoglobulins, peroxidases, fatty acids, steroids, metal-mediated systems, and cationic peptides (245), and was shown to have temporary bacteriostatic capabilities on *P. anaerobius* and *P. prevotii* in the second and third trimesters (280-281).

## 1.4 RELATIONSHIP OF *PEPTOSTREPTOCOCCUS* SPECIES WITH PELVIC INFLAMMATORY DISEASE (PID).

PID is an acute clinical condition that involves the ascension of microorganisms from the lower female genital tract (vagina, endocervix) into the upper female genital tract resulting in endometritis, salpingitis, oophoritis, parametritis, and tubal or tubo-ovarian abscesses (306). PID can lead to major reproductive sequelae that includes tubal factor infertility, ectopic pregnancy, chronic pelvic adhesive disease, and chronic pelvic pain (304). Most cases of PID (up to 75%) are caused by the sexually transmitted disease (STD) organisms *Neisseria gonorrhoeae* and/or *Chlamydia trachomatis* (152, 298). Ascension of the endogenous flora may occur by a variety of means that include ovulation, menstruation, infection with STD agents such as *Trichomonas vaginalis*, uterine contractions, sexual intercourse, attachment to spermatozoa, and by invasive means such as abortion, intrauterine device (IUD) insertion, douching, and use of obstetric instruments during childbirth (7, 119, 209, 299). Often, STD-related PID cases are polymicrobial and include the endogenous flora from the lower genital tract (31, 48). These include *Streptococcus* spp., *Escherichia coli*, *Haemophilus influenzae*, *Bacteroides* spp., *Prevotella* spp., *Porphryomonas* spp., *Peptostreptococcus* spp. and mycoplasmas. In other cases, 25-50% of PID cases have no STD organisms present but only the endogenous flora (230). BV and BV-related bacteria have been shown to be associated with an increased risk for PID (64, 66, 137, 259).

Currently, there are two accepted scenarios concerning the pathogenesis of acute PID. In the first scenario, STD organisms such as *N. gonorrhoeae* and *C. trachomatis* ascend to the upper genital tract to cause tissue damage and create an environment (raised pH, consumption of nutrients and production of waste products, lowered oxidation-reduction potential, impaired host defenses,

disrupted mechanical barriers) where the endogenous flora may ascend and proliferate (48, 53, 173, 230). In the second scenario, PID may be caused solely by the endogenous flora leading to a superinfection (65, 153, 162, 269-270). Although the incidences of PID solely due to the endogenous flora has decreased (113) (due to factors such as the use of laparoscopy over culdocentesis in sampling to reduce possible contamination, decrease in intrauterine device (IUD) use, sexual education), the endogenous flora still is believed to play a significant role by itself or in conjunction with STD agents (106, 113, 256-257, 269). *Bacteroides* spp., *Prevotella* spp., and *Peptostreptococcus* spp. have been singled out as being important contributors to the polymicrobial infection of PID because they are the most common isolated species in such cases and the cases tend to be more severe (13, 82, 152, 258).

PID cases associated with BV-related organisms tend to be more severe (abscess formation), occur in older women, in repeat patients, respond poorly to therapy, and have an increased chance of producing chronic pelvic pain, ectopic pregnancy, and involuntary infertility (83, 154, 286, 305). As shown in a rat model, the STD agents *N. gonorrhoeae* and *C. trachomatis* alone do not usually produce intraabdominal abscesses whereas *E. coli* and *Enterococcus faecalis* are producers (52). Most tubo-ovarian abscesses (TOAs) contain the endogenous flora with 63-100% being anaerobes (136, 214, 271, 282). The main organisms recovered from TOAs include *E. coli*, *Bacteroides fragilis*, *Prevotella* spp., *Peptostreptococcus* spp., and aerobic streptococci (136).

In a subcutaneous mouse abscess model, it was found that in most cases *Peptostreptococcus* spp. (*P. magnus, P. prevotii, P. asaccharolyticus, P. micros, P. anaerobius*) significantly enhanced the abscesses induced by other aerobic and anaerobic bacteria (38). Upon mixture of formalin-killed cells or capsular material of *Klebsiella pneumoniae*, or with encapsulated *Bacteroides* spp., abscess

formation occurred in 75% of the animals injected with seven of eight *Peptostreptococcus* isolates which were mostly unencapsulated. After obtaining the *Peptostreptococcus* isolates from these abscesses, they were found to be encapsulated and could induce abscess formation alone (39). These results are consistent with mechanisms of abscess formation as exemplified in a *B. fragilis* mouse model (34, 39, 52, 155).

## 1.5 VIRULENCE FACTORS OF *PEPTOSTREPTOCOCCUS* SPECIES.

Factors that contribute to the pathogenicity of peptostreptococcal infections are poorly defined. A bacterial virulence factor or determinant is an attribute or product that allows for selective advantage in establishing infection or guaranteeing its survival (63). Known or putative virulence factors of anaerobic bacteria include capsules, fimbriae, outer membrane proteins, lipopolysaccharides, metabolites, exotoxins, proteases, and other enzymes (103). A variety of known and putative virulence factors have been attributed to *Peptostreptococcus* species.

### 1.5.1 Proteases.

Proteases can be subdivided into exopeptidases which cleave the amino- or carboxy-terminus of the peptide, or endopeptidases (proteinases) that cleave internal peptide bonds (26). There are 4 classes of proteinases based on the presence of an essential catalytic residue at their active sites: serine, cysteine, aspartic or metallo (usually zinc) (12, 297). The catalytic mechanisms of serine and cysteine proteinases involves the covalent binding to their respective active residue (26). Aspartic proteinases have two aspartic residues at their centers that are involved in acid-base catalysis (26). Metalloproteinases have metal ions (usually zinc) that are believed to enhance the nucleophilicity of water and polarize the peptide bond prior to cleavage under nucleophilic attack (26).

Proteinases may also be classified according to the effects of proteinase inhibitors or enzyme

activity. However, certain proteinase inhibitors may not be specific to one class of proteinase (93). Proteinase inhibitors are proteins or peptide derivatives that prevent uncontrolled proteolysis within cells where limited proteolysis is vital for biochemical and physiological processes and protect the cell from foreign proteolytic attack (236). Proteinase inhibitors serve as pseudo-substrates by usually irreversibly combining with the active site of the protease and altering the protease by splitting a single peptide bond corresponding to its specificity requirements (139, 197).

Bacteria synthesize a large variety of proteases belonging to three out of the four classes, aspartate proteases being the exception (26). Intracellular bacterial proteases are highly specific and are involved in several biological processes. These include the removal of signal peptides from newly synthesized proteins, activation of inactive precursors, inactivation of regulatory proteins, and the breakdown of abnormal or foreign proteins for nutrition (179, 297).

**1.5.2 Pathogenic role of bacterial proteases in anaerobic infections.**

The essential role of proteases in bacteria is to obtain amino acids and peptides for survival and growth (100). Pathogenically, proteases may be involved in tissue destruction, evasion of host defenses, and changes in the operation of the immune system during infection and inflammation (288). Like other proteases, the essential function of proline aminopeptidases is to provide nutrients for growth as seen for the lactic acid bacteria (124). Proline specific proteases also play a role in breakdown of structural proteins especially collagen which is rich in proline and hydroxyproline residues (169, 250).

The possible role of proteases (elastases, collagenases, and nonspecific proteases) in female genital tract infections includes premature rupture of membranes (PROM) (167). PROM is a condition where the chorioamniotic membrane (CAM) ruptures prior to labor and occurs at term (5-

15%) and at preterm < 37 weeks (30-40%) and involves synergy between proteases of bacterial origin and proteases of neutrophils (146, 167, 247). A bacteria can form complexes with proteinase inhibitors such as $\alpha_2$-macroglobulin, which allows for increased activity of tissue collagenases (289). Certain proteases may serve as chemotactic factors to recruit inflammatory cells and may activate zymogens of other enzymes, provide nutrients to other bacteria from the host or other microbial sources, and alter the regulation of plasma proteins (167).

**1.5.3 Proteases from *Peptostreptococcus* species.**

Certain studies have implicated nonspecific proteases and collagenases from *Peptostreptococcus* species in soft tissue infections that include the female genital tract after detection on nonspecific and general substrates (127-128, 167, 264). *Peptostreptococcus* spp. produce proteases to hydrolyze protein substrates as the source of energy for growth except for *P. productus* (69, 105). Nonspecific protease production from 3 *Peptostreptococcus* spp. (*P. anaerobius*, *P. asaccharolyticus*, and *P. magnus*) from female genital tract infections has been shown (167), and collagenase and gelatinase production has been shown in *P. magnus* isolates isolated from nonpuerperal breast abscesses and diabetic foot abscesses (127-128).

**1.5.4 Proline aminopeptidases from anaerobic infections including *Peptostreptococcus* species.**

Aminopeptidases are enzymes that hydrolyze peptide bonds at or near the N-terminal end of polypeptides (240) that can be divided on the basis of their cleavage of the first peptide bond (aminoacyl-peptide hydrolases and iminoacyl-peptide hydrolases) and those that hydrolyze dipeptides from polypeptide chains (dipeptidyl peptide hydrolases). Different aminopeptides have a closely-related enzymatic activity sometimes with broad specificities. These specificities overlap, so that many natural and synthetic substrates can be hydrolyzed by more than one aminopeptidase (240).

There are two main types of proline aminopeptidases. Aminopeptidase P hydrolyzes amino-terminal residues from peptide substrates only when a prolyl residue is adjacent to it (58). Proline iminopeptidases hydrolyzes the amino-terminal prolyl residues from peptide substrates (58). Proline residues are often found in areas where the peptide chain changes direction (312). Proline residues are often present in β-turns and δ-turns both in solution and solid state and with turns often being found on the surface of the protein. This suggests that a conformational change or proteolysis at exposed prolines may be an important regulatory event (312). Bacterial proline aminopeptidases have been analyzed and sequenced from a variety of bacterial species: *E. coli* (313-314), *Lactobacillus delbrueckii* (11, 124), *Bacillus coagulans* (121); *Lactococcus lactis* (159, 193); *Streptomyces lividans* (41); and *N. gonorrhoeae* (5). Elevated levels of proline aminopeptidase have been used as a reliable marker enzyme for BV and *Peptostreptococcus* species such as *P. anaerobius* may be contributing to such activities (248). The proline aminopeptidase gene(s) of *P. anaerobius* has not been characterized.

A variety of aminopeptidase activities are present in different species of *Peptostreptococcus* (69, 182-184). Certain *Peptostreptococcus* spp. (*P. magnus*, *P. micros*, and *P. heliotrinreducens*) have been noted for their proteolytic activities (181, 185, 228, 278). Aminopeptidase activities of oral isolates of *P. micros* may account in part for the third and final stages of peptide degradation of human serum (278). Aminopeptidase activities are largely cell-associated indicating bacterial adherence to and most likely the subsequent degradation of glycoproteins (279). Increased leucine aminopeptidase activity levels in faeces from patients with Crohn's disease was observed when compared to healthy subjects (235). Patients with Crohn's disease had higher numbers of Gram-positive bacteria (*Peptostreptococcus* spp., *Eubacterium* spp., and *Coprococcus* spp.) and

*Bacteroides vulgatus* than in healthy controls (235). The increased levels of overall proteolytic activity seen may be due to several causes, both bacterial and endogenous (235).

**1.5.5 Immunoglobulin-binding and albumin-binding proteins as virulence factors in anaerobic cocci.**

Many cell wall proteins of Gram-positive cocci bind to host proteins, especially albumin and immunoglobulins in serum (91, 118, 226). These include protein A of *Staphylococcus aureus* (77), protein G of groups C and G streptococci (23, 229), protein H of group A streptococci (4, 88), protein L of *P. magnus* (21, 116, 187), and PAB (peptostreptococcal albumin-binding) protein from *P. magnus* (56). They are fibrous, elongated, and monomeric proteins found on the cell surface with the N-terminal half sticking the furthest away in the extracellular milieu with no intra- or interchain disulfide bridges (3, 118). The dominant structural domains for these cell surface proteins include a secretion signal sequence (S), binding domains consisting of repetitive amino acid sequences, a cell wall spanning domain consisting of a proline-rich region and a region which is rich in charged residues (W), and a C-terminal membrane anchor (M) (71). Prior to the C-terminal membrane anchor, a hexapeptide Leu-Pro-X-Thr-Gly-X (LPXTGX, where X- represents any aa) motif has been identified which may play a role in cell attachment of these proteins (74).

**a) Immunoglobulin-binding proteins.**

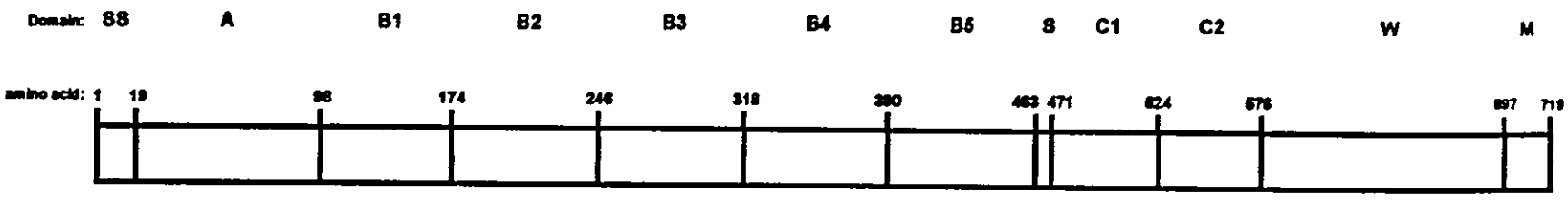
Some immunoglobulin (Ig)-binding protein capabilities are a reflection of their local milieu: IgG-binding proteins are mainly present in plasma, IgA-binding proteins (e.g. IgA receptor protein) (145) are present in bacteria found in mucosal secretions where IgA is the prominent antibody, and IgD-binding proteins of *H. influenzae* (protein D) (234) are found in the upper respiratory tract where IgD is prevalent (91). Protein A, protein G, and protein H have a strong affinity for the Fc portion

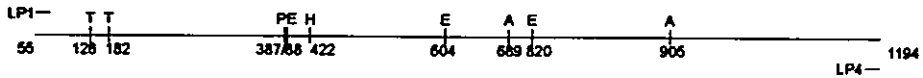
of human IgG at the Cγ2 Cγ3 domain (1, 229, 265). Protein G and Protein H also bind albumin (2, 4, 79). Often, these proteins may bind to more than one protein at once (91). A unique feature about these three proteins is that there is no sequence homology between their Ig-binding sites although they bind to the same region of IgG Fc (80).

Protein A and protein G are present in most isolates of *S. aureus* and group C and G streptococci, respectively (91, 118). Protein H is only found among the M1 serotypes of group A streptococci (4). Protein L has a unique affinity for the four major $V_L$ subgroups of the human kappa light chains of all Ig classes: $V_{KI}$, $V_{KII}$, $V_{KIII}$, and $V_{KIV}$ as well as for many mammalian counterparts such as baboon, guinea pig, mouse, rat, and pig (57, 204). Structurally, protein L has similar structural domains (Figure 1) as the other immunoglobulin-binding proteins. It has an 18 amino acid (aa) signal sequence followed by a $NH_2$-terminal domain (A) of 79 aa, and 5 repeats of 72-76 aa designated B repeats that are homologous to each other (70-80%) and serve as the Ig-binding domains. Towards the carboxy-terminus, there is an 8 amino acid spacer (S) followed by 2 repeats designated C of 52 aa of unknown function. A hydrophilic, proline-rich cell wall spanning domain (W), and the hydrophobic membrane anchor (M) follow. Preceding the hydrophobic membrane-spanning domain is the hexapeptide of leucine, proline, lysine, alanine, glycine, and serine (LPKAGS), which is similar to the consensus sequence leucine, proline, X, threonine, glycine, and X (LPXTGX, where X represents any aa) (117). $PPL_{312}$ does not bind albumin (57).

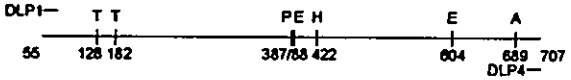
Recently, a heterologous protein L ($PPL_{3316}$) has been sequenced and analyzed (187-188). $PPL_{3316}$ is larger than $PPL_{312}$ (106 kDa versus 79 kDa; 992 aa versus 719 aa) (117, 187). The major structural differences between the two protein L structures includes the lack of sequence homology in the N-terminal regions, the number of Ig-binding domains ($PPL_{312}$ has 5 and $PPL_{3316}$ has four), and

**Figure 1.** Schematic representation of protein L ($PPL_{312}$) and restriction endonuclease map of PCR amplicon products derived from published sequences (117). Amino acid numbers are indicated at the start for each domain illustrated. The domains are as follows: the hydrophobic signal sequence (**SS**), the $NH_2$-terminal domain (**A**), the repeated units that bind immunoglobulins (**B1-B5**), the spacer region (**S**), the twin repeats of unknown function (**C**), the wall-spanning domain (**W**), and the transmembrane region (**M**).



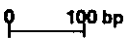




1,139 bp protein L PCR amplicon/LP1 and LP4 primers




652 bp protein L PCR amplicon/DLP1 and DLP4 primers

T - *TaqI*
P - *PstI*
H - *HindIII*
E - *EcoRI*
A - *AccI*



that only $PPL_{3316}$ has albumin-binding domains which are located towards the carboxy-termini (187).

**b) Albumin-binding proteins.**

A study of albumin-binding isolates of *P. magnus* showed that they are strongly associated with deep wound infections (56, 190). Albumin-binding by *P. magnus* isolates appears to be mediated by a heterologous collection of proteins that includes the PAB protein, $PL_{3316}$ which is a variant of $PPL_{312}$, and a 130 kDa protein (56, 135, 187). Albumin-binding proteins are also very common among group A, C, G, and L streptococci (130, 134 192). PAB is a 47 kDa protein that has the first 4/123 aa of the $NH_2$-terminal region (A) (alanine, aspartic acid, glutamic acid, and proline) that are identical to that of $PPL_{312}$, and a signal sequence (SS) (27 aa), a W region, and a M region having similarity with the corresponding regions of $PPL_{312}$ (56). The A region is followed by a C region (46 aa) showing 69% homology to the C repeats of $PPL_{312}$ of unknown function. With the W region, there is a 45 aa region that is involved in albumin-binding called the GA module (56). The GA module has 60% sequence homology to the albumin-binding domains of protein G. There is also a 103 nucleotide sequence downstream of the GA module that has 66% identity to the conjugative plasmid pCF10 from *E. faecalis* (115).

It has been proposed that cell surface binding proteins evolved by module/cassette horizontal transfer, which would maximize the spread of evolution and diversity of similar, but not necessarily identical functions in different species (91). This theory suggests that there are three modules/cassettes: the secretion signal peptide; the carboxy-terminal region involved with bacterial attachment; and a central variable region containing the protein binding domains. This theory is based on similar sequences found in these regions of different cell surface binding proteins (91). The framework regions at the nucleotide level (the amino- and carboxy-terminus) of albumin-binding proteins PAB and $PPL_{3316}$ are highly conserved when compared to $PPL_{312}$ (56, 187). This suggests

that all three proteins are recombinant proteins with cell surface binding domain(s) being acquired by horizontal transmission (56, 91, 187).

**1.5.6 Pathogenic role of peptostreptococcal protein L and other cell surface proteins.**

In the only study of its kind, > 50% of *P. magnus* isolates (4/7) from BV patients contained protein L and statistical analysis showed that protein L was correlated to BV (116). This evidence suggests that protein L may serve as a virulence factor or as a marker for pathogenicity (116). Protein L can mediate the release of histamine through affinity of the kappa light chains of membrane-bound IgE to human basophils and mast cells which may serve as a mechanism in pathogenicity (213). In contrast, there is no statistical association of protein A, protein G, and protein H to virulence since the former two are common in their hosts (118). Protein H is a member of the M-like proteins (M protein is an antiphagocytic cell surface protein of group A streptococci) (96).

At present, it has been difficult to define a pathogenic role for Ig-binding/albumin-binding proteins of Gram-positive cocci (307). However, there are many compelling theories about the pathogenic role of these proteins. The physiological properties (ionic and hydrophobic interactions) of the cell surface of streptococci cells changes upon binding to host proteins, including albumin (172). This could lead to changes in increased adherence between the bacteria and host cells affecting colonization and spreading of the organisms (251). By binding to serum proteins via their binding proteins, the bacteria may evade the host immune system of the infected host through molecular mimicry (80, 91). *P. magnus* isolates that contain protein L have been speculated to have an advantage over bacteria containing other Ig-binding proteins because the antibodies bound to protein L can still bind host cells via their Fc receptors (308).

Cell surface binding proteins may provide nutrients to the cell. Albumin is a major transport protein and could supply fatty acids and amino acids (79, 251). At low concentrations, a cysteine

proteinase from group A streptococci removes protein H and M1 protein from its cell surface which may enhance the spread of infection, e.g. when nutrients are scarce and pH is low (15).

Ig-binding proteins including protein L, protein A, and protein G can bind to the human proteinase inhibitors $\alpha_2$-macroglobulin and kininogen at different sites to which they bind proteinases (117, 252). The proteinase inhibitors bound to the cell surface may protect the bacteria from host proteases (80, 91). Alternatively, it was shown that a peptide derivative from cystatin (human cysteine proteinase inhibitor) could inhibit the cysteine proteinase of group A streptococci causing bacterial death (22). Binding of albumin by group A streptococcus increased the rate of phagocytosis by polymorphonuclear leucocytes (PMNLs) in the absence of complement and antibodies but there was prevention of killing of the cells possibly by oxygen radical quenching of the PMNLs (295).

**1.5.7 Other virulence factors of *Peptostreptococcus* species.**

*Peptostreptococcus* spp. produce a variety of other virulence factors. Bacteriocin production by certain strains of *P. asaccharolyticus* (2/4), *P. anaerobius* (3/3), and *P. magnus* (2/5) inhibited the growth of *N. gonorrhoeae* (177). As well, a human intestinal strain, *Peptostreptococcus* strain E1, produced an antibiotic-like trypsin dependent substance that was active against Gram-positive bacteria such as *Clostridium perfringens, C. difficile, C. butyricum, C. septicum, C. sordellii, Eubacterium* spp., *Bifidobacterium* spp., and *Bacillus* spp. (227). Trypsin may activate bacterial enzymes that produce the antibiotic-like substance and hydrolyze the protein inhibitors of the antibiotic-like substance (227).

*Peptostreptococcus* species produce hydrolytic enzymes that utilize host macromolecules as substrates (proteins, glycoproteins, and lipids) which may play a role in the infectious process. Certain *Peptostreptococcus* strains recovered from bovine endometritis and mastitis produce heparinase (92, 114). *Peptostreptococcus* strain 84H14S, obtained from the human oral flora, produced

hyaluronidase, which is unique in its enzyme substrate specificity because it acts not only on hyaluronic acid rapidly but it also is active against chondroitin sulfate A and C (272-273). Spent culture medium of *Peptostreptococcus* strain 84H14S inhibited the uptake of thymidine in HeLa cells independent of hyaluronidase production (274). In addition, a rat intestinal tract isolate of *P. micros* has mucin-degrading capabilities (42). *Peptostreptococcus* spp. that includes *P. anaerobius* produce phospholipase $A_2$ and phospholipase C which may play a role in premature labor (14, 166). Phospholipases of *Peptostreptococcus* spp. may contribute to the production of arachidonic acid. Arachidonic acid is involved in prostaglandin production which in turn stimulates uterine contractions (85).

In a study of oral isolates, *P. micros* had a rough morphotype rather than the more common smooth morphotype (292). These rough morphotype isolates could be distinguishable from the smooth morphotypes by being more hemolytic, more hydrophilic, and contained fibrillar structures on its surfaces which may play a role in adhesion (292). Crystalline surface proteins (S-layer) have been isolated from *P. asaccharolyticus* (170), *P. anaerobius* (126), and *Peptostreptococcus* AHC5155, a strain that resembled *P. magnus* (147). Although the role of S-layers in virulence is not known, it has been shown to be a true virulence factor in *Aeromonas hydrophila* and *Campylobacter fetus* (126).

## 1.6 BACTERIAL TYPING.

One main reason for carrying out typing studies is to determine whether isolates with putative virulence factors are clonally related since most bacterial pathogens are clonal in nature (8). Typing systems are based on the premise that clonally related isolates share characteristics by which they can be differentiated from unrelated isolates (8, 156). For each typing system, three parameters may be examined: typeability, reproducibility, and discriminatory power. Typeability refers to a method where

a percentage of bacterial isolates examined is given a recordable reaction (8, 110, 120). Reproducibility of a method refers to its ability to produce the same result when the test is performed repeatedly. Reproducibility may be affected by variation in the culture conditions and the structural and genetic make-up of the number of examined isolates (110, 120, 156). Discriminatory power of a method refers to its ability to differentiate unambiguously among unrelated strains (25, 110, 120, 156, 220). It is determined by the number of types identified by the method and the relative frequencies of these types (25).

A clone is a descendent from a common parental cell. To successfully identify a clone, the genetic stability of the organism, the selective pressure of the environment, and the discriminatory power of the typing method come into play (62, 207). However, clonality is more of a relative than an absolute concept (62). A bacterial species is "a collection of strains that share many features in common and differ considerably from other strains" (263) and are monophyletic, i.e., they include all the descendents from one population (158). DNA-DNA relatedness with strains that have about 70% or more similarity are considered to be one species (300). Also, using common physiological, biochemical, and morphological characteristics, an 80% similarity between strains defines a species (215). The type strain serves as the reference strain of the species and is the permanent example of the species (263).

The two major classes of typing methods are phenotypic and genotypic (8). Phenotypic typing methods take into account of biochemical, physiological, and biological characteristics of the strains (62). Phenotypic typing methods include biotyping, antibiogram determination, bacteriophage typing, serotyping, electrophoretic protein typing and immunoblotting, and multilocus enzyme electrophoresis (MLEE) (8, 62). There are several major disadvantages concerning phenotypic methods. Most phenotypes are under environmental selective pressure and therefore may vary (17, 62). Many of the

phenotypic methods were developed for a certain genus or species, and are thus limited in their scope of use for other organisms. For example, in serotyping, high quality commercial reagents are used to prepare specific typing antibodies and their use is limited to reference and research laboratories (8). Other disadvantages include variable expression of those characteristics under examination, insufficient discrimination to yield useful epidemiological information, and non-typeability of many isolates (120).

Phenotypic methods that have been applied to various *Peptostreptococcus* species include analysis of the murein type of the cell wall (302), GLC analysis of major cellular fatty acids and volatile fatty acids (133), whole cell protein electrophoresis (275), high pressure liquid chromatographic analysis of amino acid utilization and production of metabolites in a defined chemical medium (95), serological analysis of cell surface components (51, 253-254), and conventional biochemical characterization (133, 311). Although these strategies have provided additional information in the classification of *Peptostreptococcus* spp., there are some drawbacks that include limited powers of discrimination at the species level, equipment which is costly and inaccessible for use in routine clinical laboratories, and lack of interlaboratory standardization (133, 183, 253).

Genotyping methods involves DNA analysis of chromosomal and/or extrachromosomal genetic elements and includes plasmid profile analysis, chromosomal DNA restriction endonuclease analysis, ribotyping, pulsed-field gel electrophoresis (PFGE), typing systems applied to polymerase chain reaction, and nucleotide sequence analysis (8). Most of the genotyping methods involve the examination of band fragments in a gel or on a membrane that are then compared for similarity (219). The advantages of genotyping methods are as follows: DNA can usually be extracted from bacteria allowing for all strains to be typed, there is uniformity in the analysis of the DNA under examination,

many of the procedures used do not need species-specific reagents, and it is possible to study neutral markers, which at least on chromosomal DNA are rather stable (17, 112, 222). Many of the genotyping methods such as ribotyping, PFGE, PCR-based typing systems, and nucleotide sequence analysis have been developed in the past 15 years or so and are likely to play a more significant role in clinical strain characterization (151).

There have been very few studies on typing of *Peptostreptococcus* species. Plasmid analysis may be useful for epidemiological studies in a restricted time and space because plasmids may be spontaneously lost from or acquired by a host strain (8). Plasmid analysis is not very discriminating and different strains may harbour nonidentical plasmids of the same size and differences in mobility of open and closed plasmids. These problems can be overcome by digesting the plasmids with restriction endonucleases (8). Plasmid DNA analysis was performed on 32 *Peptostreptococcus* isolates and only 3 isolates were found to contain plasmids, therefore plasmid analysis was found not to be discriminating (200).

Restriction endonuclease digestion of chromosomal DNA (micro REA) results in patterns that are complex and may result in up to 200 fragments of different size ranges (0.5- 50 kb) (287). Strain-to-strain variations in fragment sizes are called restriction fragment length polymorphisms (RFLPs) and usually are due to a point mutation, insertion, or deletion resulting in the presence or absence of a restriction site at a given position in the genome (17).

Ribotyping involves the Southern blot analysis where strains are characterized for their RFLPs associated with rRNA operon(s) (266). The number of positive hybridization bands is usually small enough (7 to 12 on average) to allow for easy analysis of the banding patterns compared to chromosomal REA (222). Ribotyping has provided excellent discrimination and reproducibility for the epidemiological typing of a variety of bacterial species that include *Legionella pneumophila*

serogoup 1 (243), *Staphylococcus epidermidis* (16), *Helicobacter pylori* (175), *Neisseria meningitidis* (78), and *N. gonorrhoeae* (141). The choice of restriction endunuclease(s) is vital to the rRNA hybridization patterns of the DNA obtained when probed with the DNA-based rRNA cistrons after labelling (94, 220). The reliability and sensitivity of the ribotype patterns is enhanced when 2 restriction endonucleases are used rather than one (138).

Most bacterial *rrn* operons consist of the promoter followed by 5'- 16S rRNA - 23S rRNA - 5S rRNA genes -3' and may vary in nature and copy number depending on the species. In *E. coli*, there are 7 *rrn* operons with a spacer region between the 16S and 23S rRNA genes that encodes for tRNA genes and other variable sequences (176). The 16S and 23S rRNA genes are conserved and can be used as a common probe for typing (detection of RFLPs of rRNA genes or ribotyping) most bacterial species (17). Plasmid pKK3535, a recombinant plasmid of pBR322, contains the *rrn*B operon of *E. coli* (40). The labelled 7.2 kb *Bam*HI-*Pst*I fragment of pKK3535 that contained the *rrn*B operon was a successful probe for intra- and inter-species differentiation of *Peptostreptococcus* species (*P. anaerobius*, *P. asaccharolyticus*, *P. magnus*, *P. micros*, and *P. prevotii* ) that included type strains and isolates of unknown clinical origin after digestion of their genomic DNA with *Eco*RI and *Hin*dIII (201).

PFGE (macro REA) was first applied on fractionated chromosomal DNA of *Saccharomyces cerevisiae* (249). Conventional agarose gel electrophoresis has limitations because DNA fragments > 20 kb are difficult to resolve and size (165). PFGE does not have this problem because it utilizes at least two sets of electrodes to periodically alter the direction of the electrical field in which the DNA is moving (206). Every time the field is alternated, the DNA fragments must reorient before moving forwards (206). The time for reorientation is dependent on the size of the DNA fragment with smaller sized DNA fragments changing direction and migrating faster (206). In most cases, PFGE

analysis resolves the entire genome into a simpler pattern of 5-20 fragments after digestion with rare-cutting restriction endonucleases whereas bacterial REA produces digestion patterns with many bands making comparisons difficult (9, 156). These fragments typically range from about 10 kb to 800 kb in size (9, 156). Theoretically, all bacterial isolates should be typeable by PFGE with the results being reproducible (156). PFGE has been highly discriminating and comparable or even better than other typing methods for *E. coli* (9), enterococci (189), staphylococci (225), *Pseudomonas aeruginosa* (221), and *Mycobacterium avium* (10). Application of PFGE to *Peptostreptococcus* spp. has not been reported to date.

PCR was first developed to detect sickle cell hemoglobin genes in humans (237). The PCR reaction involves the *in vitro* amplification of multiple copies of a segment of a gene or DNA using oligonucleotide primers which bind to opposite strands, flanking the target sequences (291). In each cycle, there is DNA denaturation, annealing of the primers to the DNA template, and DNA polymerase extension across the template. The reaction product is double stranded DNA with termini corresponding to the 5' ends of the primers used and is used as the template for the next cycle (283). After 20-30 cycles, this exponential reaction allows for the possible amplification of the original DNA segment to be around a million-fold (291).

Polymerase chain reaction-restriction endonuclease analysis' (PCR-REA) discriminatory power varies on the bacterial species loci and choice of restriction endonucleases (8). In certain cases, the digest patterns is species specific but does not identify individual isolates (276), whereas in other cases, PCR-REA is moderately discriminating (87). PCR-REA has been applied to various bacterial species: *Mycobacteria* spp. (276), *S. aureus* (87), *H. pylori* (81), *Mycobacterium* spp. (231), and *Rochalimaea* spp. (157). Strain differentiation can be increased by digesting with several different restriction endonucleases (81, 157). PCR-REA is highly reproducible, sensitive, versatile in terms of

experimental conditions, and it allows a large number of strains to be analyzed at once (290).

## 1.7 IDENTIFICATION OF *PEPTOSTREPTOCOCCUS* SPP.

Although *Peptostreptococcus* species are associated with a variety of female genital tract infections, their role in such infections is not clear (38, 83, 198). Part of the problem in ascertaining the pathogenic potential of *Peptostreptococcus* species concerns the identification and taxonomic classification of clinical isolates (34, 154). The accepted techniques for identifying anaerobic bacteria include gas-liquid chromatography (GLC) and biochemical tests used in the system of the Virginia Polytechnic Institute (VPI) (104, 267). However, GLC is expensive, time-consuming, and often inaccessible to the routine clinical laboratory (122). With the introduction of new isolates, phenotypic characterizations based on pre-formed enzyme kits, such as the rapid ID32A, and whole-cell composition by pyrolysis mass spectrophotometry can be used to resolve classification anomalies of *Peptostreptococcus* spp. based on the VPI method (180). Furthermore, because most *Peptostreptococcus* are asaccharolytic (69), species characterization is based on negative results (105, 182).

Recently, there have been several papers published denoting changes in the taxonomic classification of *Peptostreptococcus* (68, 142, 180). This is due to improvements in anaerobic culture conditions, the introduction of pre-formed enzyme kits such as the API ZYM and AN-IDENT systems (API, La Balme Les Grottes, Montalieu Vercieu, France), the RAPID ANA system (Mercia Diagnostics, Guildford, Surrey) and the ATB 32A system (bioMérieux, Basingstoke, Hands) (142, 182-184) that includes tests for various oxidases, peptidase, and sugar fermentation reactions, and the use of other techniques such as 16S rRNA sequencing, and pyrolysis mass spectrophotometry (143, 150).

Comparative analysis of 16S rDNA sequences from *Peptostreptococcus* species has been a

useful strategy in taxonomic work (143). 16S rDNA sequences showed that *P. productus* clustered with the genus *Ruminococcus* and has been proposed to be placed in this genus (67) whereas another group has suggested that this would be premature since the genus *Ruminococcus* itself is too heterogeneous (309). 16S rDNA sequence comparison has also shown that *P. anaerobius* clusters with *Clostridium* species (212).

Alternate taxonomic methods have been used in the classification of *Peptostreptococcus* spp. These include DNA-DNA and DNA-rDNA homology (111), guanine-plus-cytosine content of DNA (69), and various phenotypic methods listed earlier with similar drawbacks (183).

In many previous studies, *Peptostreptococcus* isolates from female genital tract infections tended to be grouped rather than identified to the species level. This may neglect the potential pathogenic relationships between certain bacterial species (101) and clearly shows the importance of identifying *Peptostreptococcus* to the species level.

**1.8 RATIONALE AND OBJECTIVES.**

*Peptostreptococcus* species are part of the normal flora and also are associated with clinical pathologies that include female genital tract infections. One means to distinguish pathogenic *Peptostreptococcus* isolates from members of the normal flora is to identify and/or characterize virulence factors. Several studies showed that proteolytic enzymes from *Peptostreptococcus* species may be important in the pathogenesis of soft tissue infections (127-128, 167, 228, 278). Both proteases (nonspecific and collagenases) and aminopeptidases (248) may have been attributed to such activities especially in oral infections (278-279). However, in the above studies, no proteolytic genes have been sequenced and the nature of the proteolytic activity has not been quantified. This may be important in determining the type of proteolytic enzymes involved.

The "gold" standard for identification of anaerobic bacteria is the VPI method. There are

problems with the VPI method that include difficulties in identification of various *Peptostreptococcus* isolates and it is difficult to perform. An alternate method is to use pre-formed enzyme kits such as the recently introduced rapid ID32A kit (bioMérieux Vitek Inc., Hazelwood MI.), which includes twelve aminopeptidase reactions.

There is little information on the genetic make-up of pathogenic strains of *Peptostreptococcus* species. Two putative virulence factors include protein L and albumin-binding proteins. Previously, protein L was identified in 4 of 30 *P. magnus* isolates from Swedish BV patients (116), while 15 of 36 *P. magnus* isolates characterized in another study bound albumin (190). There have been no published reports on the clonality of *P. magnus* isolates containing these or other virulence factors and there is a need to determine whether protein L is present in other *P. magnus* isolates from different female genital infections.

My first objective was to isolate and characterize protease genes from *Peptostreptococcus* isolates. *Hypothesis*: The proteolytic activity of *Peptostreptococcus* isolates is due to proteinases and not nonspecific proteases such as aminopeptidases.

My second objective was to determine the nature of the proteolytic activity. The rapid ID32A kit can be used to screen for *Peptostreptococcus* isolate's aminopeptidase activity and to see if it is correlated to proteolytic activity (gelatin hydrolysis). Semi-quantitative analysis of the proteolytic activity using nonspecific and general substrates may provide information on the type of proteolytic enzymes involved with *Peptostreptococcus* isolates. *Hypothesis*: The proteolytic activity of *Peptostreptococcus* isolates is due to an array of positive aminopeptidase reactions observed.

My third objective was to use the aminopeptidase profiles from the rapid ID32A kit along with the VPI method to provide new insights in the taxonomic classification of *Peptostreptococcus* (mainly from the female genital tract). *Hypothesis*: Aminopeptidase profiles are a reliable method to

classify *Peptostreptococcus* species as compared to the VPI method.

My fourth objective was to determine the genetic make-up of protein L-containing and albumin-binding isolates of *P. magnus* using three genotyping methods (PCR-REA, ribotyping, and PFGE) and to determine if protein L is found in other *P. magnus* isolates from different female genital tract infections. *Hypothesis*: Protein L-containing and albumin-binding *P. magnus* isolates have a clonal structure.

My fifth objective was to determine the sensitivity and specificity for the three genotyping methods (PCR-REA, ribotyping, and PFGE) based on typeability, reproducibility, and discriminatory power and to determine if the UPGMA method is effective in clustering particular phenotypes of *P. magnus* isolates. *Hypothesis*: PFGE is the best genotyping method compared to PCR-REA and ribotyping and UPGMA is an effective method for clustering particular phenotypes of *P. magnus* isolates.

# 2. MATERIALS AND METHODS

## 2.1 BACTERIAL ISOLATES.

A total of one hundred and forty-three different *Peptostreptococcus* isolates were used in this thesis (Table 1 and Appendix B, Table 20). A subset of one hundred *Peptostreptococcus* isolates comprising 5 species were used for identification and aminopeptidase detection using the rapid ID32A kit (Appendix B, Table 20, Study 1 and 2). These included the 5 type strains: *P. anaerobius* ATCC 27337, *P. asaccharolyticus* ATCC 14963, *P. magnus* ATCC 15794, *P. micros* ATCC 33270, and *P. prevotii* ATCC 9321; 68 isolates from the female genital tract; 5 isolates from breast abscesses (127-128); 2 isolates from skin abscesses (70); and 20 isolates from unknown clinical origin (201). Twenty isolates were *P. anaerobius*, 25 isolates were *P. asaccharolyticus*, 24 isolates were *P. magnus*, 19 isolates were *P. micros*, and 12 isolates were *P. prevotii* (Appendix B, Table 20, Study 1 and 2).

Thirty-two epidemiologically unrelated *P. magnus* isolates were used in the clonal analysis study (Appendix B, Table 20, Study 3). Fifteen of the 32 isolates had not been used in the above studies. These included isolates from various female genital tract conditions such as mucopurulent cerevicitis (MPC9814), vaginal hysterectomies (VH9072), healthy patients from a tampon study (T9610) (47) and septic abortions (SA652A, SA1339F, SA2005C) (49) from A. Chow (Vancouver General Hospital, Vancouver, British Columbia). Isolates 595, 564, and 624 were obtained from Swedish patients with bacterial vaginosis (108, 116); isolates KA, BL 3410, and BL 3431 were obtained from different Swedish female patients having either an abnormal vaginal flora, a pelvic abscess or a breast abscess, respectively; and isolates BL 1116, BL 1730, and BL 2771 were

**Table 1.** Origin of isolation and source of 143 *Peptostreptococcus* isolates.

| Origin of isolation | No. of isolates in 6 species[a] | | | | | | Supplier (Reference) |
|---|---|---|---|---|---|---|---|
| | *P. anaerobius* (n= 26) | *P. asaccharolyticus* (n= 32) | *P. magnus* (n= 41) | *P. micros* (n= 25) | *P. prevotii* (n= 17) | *P. productus* (n= 2) | |
| Type strain | 1 | 1 | 1 | 1 | 1 | 0 | ATCC[b] |
| Bacterial vaginosis | 0 | 0 | 5 | 0 | 0 | 0 | ATCC[c] (191); E. Holst (108) |
| Breast abscess | 0 | 0 | 1 | 5 | 0 | 0 | C. Edmiston (127-128); E. Holst (108) |
| Cesarian section | 4 | 8 | 1 | 2 | 2 | 0 | A. Chow (310) |
| Intrabdominal abscess | 0 | 0 | 1 | 0 | 0 | 0 | E. Holst (108) |
| Mucopurulent cerevicitis | 2 | 3 | 2 | 0 | 0 | 0 | A. Chow |
| Ovarian abscess | 0 | 0 | 2 | 0 | 0 | 0 | T. Ezaki (70) |
| Pelvic abscess | 0 | 0 | 1 | 0 | 0 | 0 | E. Holst (108) |
| Pelvic inflammatory disease | 2 | 6 | 2 | 2 | 4 | 0 | A. Chow (48) |
| Scrotal abscess | 0 | 0 | 1 | 0 | 0 | 0 | E. Holst (108) |
| Septic abortion | 11 | 4 | 8 | 3 | 3 | 0 | A. Chow (49) |
| Skin abscess | 0 | 0 | 2 | 0 | 0 | 0 | T. Ezaki (70) |

**Table 1 continued.**

| Origin of isolation | No. of isolates in 6 species[a] | | | | | | Supplier (Reference) |
|---|---|---|---|---|---|---|---|
| | *P. anaerobius* (n= 26) | *P. asaccharolyticus* (n= 32) | *P. magnus* (n= 41) | *P. micros* (n= 25) | *P. prevotii* (n= 17) | *P. productus* (n= 2) | |
| Tampon | 2 | 4 | 7 | 6 | 0 | 0 | A. Chow (47) |
| Unknown | 3 | 6 | 3 | 2 | 6 | 0 | P. Ewan (201) |
| Vaginal hysterectomy | 1 | 0 | 3 | 4 | 1 | 2 | A. Chow |
| Wounds | 0 | 0 | 1 | 0 | 0 | 0 | E. Holst (108) |

[a] See Appendix B.

[b] American Type Culture Collection, Rockville, USA.

[c] Protein L-containing *P. magnus* isolate deposited by L. Björck.

obtained from Swedish males with an intraabdominal abscess, a scrotal abscess, and a deep arm wound, respectively (Table 1).

*Porphyromonas gingivalis* W83 was used as the reference strain for tne semi-quantitative analysis of protease activity because it contains at least 4 proteinase genes (2 trypsin-like proteases, a collagenase, and a cysteine proteinase) (29, 76, 131, 223) and there is no comparable control for protein substrate profiles of *Peptostreptococcus* species. *P. gingivalis* W83 was grown anaerobically on Todd-Hewitt agar plates supplemented with laked human blood (50 ml/l), hemin (10 µg/ml), and vitamin K (1 µg/ml) (29).

**2.2 GROWTH CONDITIONS.**

Isolates were revived either from lyophilized (2% skim milk or Brain Heart Infusion (BHI)) cultures by growth in Robertson's Cooked Meat medium or from storage at -70°C in BHI with 15% (v/v) glycerol by plating onto Columbia blood agar (CBA) (Quelab, Montréal, Québec) supplemented with 5% (v/v) sheep blood, vitamin K (1 mg/l) and 0.1% haemin. The blood agar plates were incubated for 24 hr at 37°C in an anaerobic chamber (5% $CO_2$, 5% $H_2$, 90% $N_2$).

**2.3 CONFIRMATION OF ISOLATE IDENTITY.**

All *Peptostreptococcus* isolates were identified by various collaborators using the VPI method. Nine clinical *Peptostreptococcus* isolates from C. Edmiston and T. Ezaki upon receipt were reconfirmed for identity by the VPI method (Chris Cooper, National Laboratory of Bacteriology, LCDC, Ottawa, Ontario) (104, 267). Previously, the five ATCC type strains and 20 clinical isolates of unknown origin from P. Ewan were reconfirmed for identity by the VPI method (201). The 100 female genital tract clinical *Peptostreptococcus* isolates from A. Chow (Department of Medicine, University of British Columbia, Vancouver, British Columbia) were originally identified by the VPI

method (47-49, 310). The ten *P. magnus* isolates *from* Sweden were identified by E. Holst by the VPI method (108). Reconfirmation of the original identification for 35 *Peptostreptococcus* isolates (5 ATCC type strains, 24 isolates from the female genital tract, and 6 isolates of unknown clinical origin) (Appendix B, Table 20, Study 1 and 2) was performed by Dr. Shupeng Chou (Ontario Provincial Health Laboratories, Ottawa, Ontario) by analysis of volatile fatty acids from cultures inoculated in Robertson's Cooked Meat broth for 48 hours using GLC with a fused-silica capillary column (104, 178, 267). Assessment of culture purity was by Gram stain and aerobic incubation.

**2.4 IDENTIFICATION AND AMINOPEPTIDASE DETECTION USING RAPID ID32A.**

The rapid ID32A system was used without prior information concerning the identities of the isolates. The rapid ID32A system (bioMérieux Vitek, Inc., Hazelwood, MI.) comprises 3 empty cupules as negative controls and 29 cupules containing the following dehydrated enzyme substrates for 6 tests used in Bergey's manual for differentiation of *Peptostreptococcus* (105): urease (URE), alpha glucosidase (αGLU), beta glucurunidase (βGUR), reduction of nitrates (NIT), indole production (IND), alkaline phosphatase (PAL) and 23 other enzymes including an array of 12 aminopeptidase reactions (122): arginine dihydrolase (ADH), alpha galactosidase (αGAL), beta galactosidase (βGAL), beta galactosidase 6 phosphate (βGP), beta glucosidase (βGLU), alpha arabinosidase (αARA), beta N-acetyl-glucosaminidase (βNAG), mannose fermentation (MNE), raffinose fermentation (RAF), glutamic acid decarboxylase (GDC), alpha fucosidase (αFUC), arginine arylamidase (ARG), proline arylamidase (PRO), leucyl glycine arylamidase (LG), phenylalanine arylamidase (PHE), leucine arylamidase (LEU), pyroglutamic acid arylamidase (PYR), tyrosine arylamidase (TYR), alanine arylamidase (ALA), glycine arylamidase (GLY), histidine arylamidase (HIS), glutamyl glutamic acid arylamidase (GG), and serine arylamidase (SER). An organism was

identified using the criteria suggested by the manufacturer (bioMérieux Vitek, Inc.) which is based on grouping positive and negative reactions into an 8-digit code and comparing it to a pre-established data base. Included in the product database of rapid ID32A were the following *Peptostreptococcus* species: *P. anaerobius*, *P. asaccharolyticus*, *P. indolicus*, *P. magnus*, *P. micros*, and *P. prevotii*. For isolates from the 4 *Peptostreptococcus* species excluding *P. prevotii*, strips were inoculated in duplicate according to manufacturer's instructions. Reactions were visually described as being positive, weakly positive, variable (very weak positive) or negative based on colorimetric indicators. Profiles of *P. prevotii* isolates were repeated up to six times because certain pre-formed reactions were inconsistent due to very weak reactions for some tests. The final identification was recorded based on the majority result from the profiles where four of six replicates gave the same colour reactions.

**2.5 GELATIN HYDROLYSIS.**

Gelatin hydrolysis by *Peptostreptococcus* isolates was determined as described previously (104). Gelatin tubes were inoculated with plate cultures and grown anaerobically at 37°C for one week, and then placed at 4°C for 45 min. Strong gelatin hydrolysis tubes were recorded as those that did not solidify at 4°C after 45 min. Solidified gelatin tubes were left at room temperature and those that required half the time for liquefaction compared to a negative (uninoculated) control were recorded as weak hydrolysis.

**2.6 PROTEOLYTIC PROFILES FOR GENERAL AND NONSPECIFIC SUBSTRATES.**

Profiles on general and nonspecific proteolytic substrates was performed to determine the nature and extent of proteolysis. Proteolytic profiles for ten of eleven general and nonspecific substrates (azocoll, azocasein, benzoyl-arginine-*p*-nitroanilide, bovine serum albumin, casein, casein

yellow, collagen (autoclaved), fibrinogen, gelatin, and glycyl-L-proline-*p*-nitroanilide) of 4 clinical isolates of *P. magnus* (Gifu 7651, 7653, 7723, and 7735) were determined by D. Mayrand using the methods described (29) except for collagen (160).

The collagenase assay performed by D. Mayrand was modified from the original procedure (160): 100 µl of cell suspension with 100 µl of collagen (Sigma Chemical Co., St. Louis, MO., C-3511) (0.1% in 0.01% acetic acid) was incubated for 4 h at 37°C. Fifty µl of 0.04 N phosphotungstic acid and 50 µl of 2 N HCl was added to the mixture, incubated at 30 min. at room temperature, and centrifuged. The $OD_{280}$ of the supernatant was determined. The proteolytic activity for the 11 substrates were compared to those of *P. gingivalis* W83. The *P. magnus* cell concentrations ($OD_{600}$= 2.0) in 100 mM Tris, pH 8.0 were 10 times that of *P. gingivalis* W83.

**2.7 ALBUMIN-BINDING ASSAY.**

Albumin-binding assays on all isolates used in genotyping studies were performed by E. Holst using the method of de Château and Björck (56). Bacteria were suspended, heat-killed (80°C, 5 min.), and washed in phosphate-buffered saline (PBS) containing 0.02% $NaN_3$ and 0.5% Tween 20. Bacterial suspensions of different concentrations in a volume of 100 µl were mixed with 900 µl of *S. aureus* strain L603 ($10^9$ bacteria/ml). Two hundred µl of bacterial suspension were then incubated with $10^4$ cpm of $^{125}$I-labelled human serum albumin for 30 min. Cells were spun down, and the radioactivity of the pellet was measured in a γ-counter and expressed as percentage of added radioactivity (56).

**2.8 PURIFICATION OF GENOMIC DNA.**

*Peptostreptococcus* genomic DNA was obtained using a modified version of the Ng and Dillon method for genotyping analysis (201). Cells were grown anaerobically on BHI agar plates with

supplements for 2-3 days at 37°C (201). Confluent growth from each plate was inoculated into 25 ml Robertson's cooked meat medium and grown overnight for 24 hr. Five ml of the overnight culture was used to inoculate a 25 ml Peptone Yeast Extract (PY) broth (201). After 24 hr, the PY broth culture was inoculated into 225 ml PY broth. Penicillin G was added to the 24 hr culture (0.01 units/ml). Cells were grown for a further 2-4 hr to weaken the cell walls for lysis. Cells were spun down at 6,000 rpm for 5 min. and the supernatant was decanted. Five ml of TES buffer (10 mM Tris, pH 8.0; 1 mM EDTA, 25% sucrose) was added to resuspend the cells. Achromopeptidase (1,000 units/ml; Wako, Richmond, VA.) and lysozyme (3 mg/ml) were added to lyse the cells at 37°C for 30 min. prior to lysis with proteinase K (0.2 mg/ml) and SDS (0.5%, w/v) for another 37°C for 30 min. incubation. An equal volume of chloroform:isoamyl alcohol (24:1 v/v) was used to deproteinate the lysate and after mixing, the mixture was centrifuged at 8,000 rpm for 10 min. at 4°C. One-tenth vol of 3 M Na acetate (pH 4.5) was added to precipitate the DNA by 2 volumes of 95% (v/v) of ethanol at - 20°C.

## 2.9 DETECTION AND RESTRICTION ENDONUCLEASE ANALYSIS OF PROTEIN L SEQUENCES.

Detection of protein L gene sequences from *P. magnus* isolates was performed by the PCR and using protein L specific primers LP1 (5'-GCTGATGAACCTATTGATCTT-3') and LP4 (5'-TTCTTCTGGTTTTTCGTCAAC-3') (General Synthesis and Diagnostics, Toronto, Ontario) (117). LP1 corresponded to position 55-75 and LP4 corresponded to position 1174-1194 of the protein L gene (117). The PCR protocol of Kastern *et al.* (117) included 1 cycle at 95°C for 5 min., 25 cycles at 95°C for 1 min.; 55 °C for 1 min.; and 72°C for 2 min., and 1 cycle at 95°C for 1 min.; 55°C for 1 min.; and 72°C for 10 min. and was modified to include a 10 min. hot start at 80° C and a higher

primer concentration of 0.5 μM using the DNA thermal cycler 480 (Perkin Elmer Cetus, La Jolla, CA.). Degenerate protein L primers DLP1 (5'-GC(T/A)GA(T/C)GAACC(T/A)AT(T/C)GA(T/C)-CTT-3') and DLP4 (5'-TTCTTC(T/A)GG(T/C)TTTTC(G/A)TC(A/T)AC-3') were synthesized (University of Ottawa Biotechnology Research Institute, Ottawa, Ontario) based on the codon usage of the protein L gene. PCR was performed as above with the addition of 2 cycles for low stringency (132) annealing at 1 min. at 94°C; 1 min. at 35 °C; and 4 min. at 72 °C; DLP1 and DLP4 primers at a concentration of 2 μM, and 1 μg of template DNA. The protein L PCR amplicons obtained using either set of primers were digested with one of *Acc*I, *Eco*RI, *Hin*dIII, *Pst*I or *Taq*I (Boehringer Mannheim Canada, Laval, Quebec). Fragments were resolved on either 4% AmpliSize agarose gels (Bio-Rad, Hercules, CA.) or 3% MetaPhor™ agarose (FMC BioProducts, Rockland, ME.) in 1X Tris-acetate EDTA (TAE) buffer (40 mM Tris-acetate, pH 8.0; and 1 mM EDTA) at 3.3 V/cm with either a 1 kb ladder (12.216-.075 kb; BRL/GIBCO; Gathersburg, MD.) or an AmpliSize standard (50 bp to 2.0 kb; Bio-Rad) as the molecular size marker. Three percent MetaPhor™ agarose, which is optimal for separation of DNA fragments from 150-700 bp, proved to be the better gel matrix in these studies; bands that may have appeared as doublets, and most small sized bands (< 300 bp), that were poorly visible on 4% AmpliSize agarose, were resolved on 3% MetaPhor™ agarose gels. DNA fragment sizes in kb were based on electrophoretic mobility relative to appropriate DNA markers was determined with a PC compatible program DNAFRAG (244). Amplicons were confirmed by Southern blot hybridization using an internal 21 bp protein L sequence designated LP5 from position 149-165 of the protein L gene (5'-GAAGAACCAAAAGAAGAAGTT-3') (117). This probe was labelled at 37°C for 15 min. nonradioactively at a concentration of 100 pM using the DIG oligonucleotide tailing kit (BMC). One μl of glycogen solution and 200 μl of 0.2 M EDTA, pH 8.0

were mixed, and 2 µl of the mixture was added to stop the labelled reaction. The labelled oligonucleotide was precipitated with 2.5 µl of 4 M LiCl and 75 µl 95% ethanol at - 70°C for 30 min. and centrifuged. This process was repeated and the labelled probe was precipitated overnight at - 20°C. The pellet was dried, washed with 70% ethanol, and dissolved in $ddH_20$. Hybridizations were performed at $T_m$ -5° C. Post-hybridization washes were performed twice for 5 min. at room temperature in 2 X SSC (1 X SSC contains 150 mM NaCl and 15 mM sodium citrate, pH 7.0) and 0.1% SDS and twice for 15 min. at 68°C in 0.1 X SSC and 0.1% SDS. Sizes of amplicons and fragments of protein L PCR-REA profiles were based on the use of the 1 kb ladder (BRL/GIBCO) and the AmpliSize marker (Bio-Rad) and on averages of 3 replicates.

**2.10 PCR AND RESTRICTION ENDONUCLEASE ANALYSIS OF THE 16S rRNA GENE.**

The partial 16S rRNA gene in *P. magnus* isolates was amplified using the PCR protocol and universal primers fD1 and rD1 designed by Weisburg *et al.* (301), with the linker sequences containing restriction sites for cloning excluded. The PCR protocol involved 25 cycles at 95°C for 2 min.; 42°C for 30 sec; and 72°C for 4 min. and 1 cycle at 95°C for 2 min.; 42°C for 30 sec; and 72°C for 20 min. Amplicons were analyzed by restriction endonuclease digestion with *Alu*I, *Hin*fI, *Hpa*II, *Sty*I, and *Taq*I (BMC) and *Sau*3AI (Pharmacia Biotechnology, Baie D'Urfé, Quebec). Agarose gel electrophoresis conditions were similar to those for protein L-based PCR-REAs (see above). Sizes of amplicons and fragments were based on using the AmpliSize marker (Bio-Rad) and the 1 kb ladder (BRL/GIBCO) and on averages of 5 replicates.

**2.11 RIBOTYPING.**

Genomic DNA from *P. magnus* isolates was separately digested with *Eco*RI and *Hin*dIII as previously described (201). Digested genomic DNA was resolved on a 1% agarose gel in 1X TAE

buffer, pH 7.8 at 0.83 V/cm to maximize pattern fragment separation and fragments were transferred to nylon membranes (Amersham, Oakville, Ontario) as described previously (46). The 7.2 kb *Bam*HI-*Pst*I fragment of plasmid pKK3535, which contains the *rrn*B operon of *E. coli* (40), was radiolabelled with [$\alpha$-$^{32}$P]dGTP using the random primer DNA labelling system (Megaprime, Amersham) and used to probe the Southern blots. The labelled reaction took 10 min. The labelled probe was separated from unincorporated [$\alpha$-$^{32}$P]dGTP as follows: 50 µl of 4 M ammonium acetate, pH 4.5 was added and then the labelled DNA was precipitated by adding 200 µl of ethanol at - 70°C for 15 min. The tubes were transferred to a heating block at 37°C for 2 min. and then centrifuged for 15 min. The supernatant was decanted and the pellet was washed once in 0.5 ml of 0.67 M ammonium acetate, pH 4.5 and 67% ethanol at room temperature, and then once in 90% ethanol. The pellet was dried and dissolved in 100 µl TE buffer. The specificity of the probe (5 X $10^5$ cpm/µl) was determined by liquid scintillation counting using a 10 µl aliquot. Hybridizations were performed at 58°C in 10% polyethylene glycol, 1.5 X SSPE (0.225 M NaCl, 15 mM $NaH_2PO_4 \cdot H_2O$, 1.5 mM EDTA, pH 7.4) (239), and 7% SDS. Ribotyping was repeated 3 times.

**2.12 MACRO RESTRICTION DNA ANALYSIS BY PFGE.**

*P. magnus* isolates were grown on biphasic medium which comprised 500 µl pre-reduced cooked meat medium (Unipath, Nepean, Ontario) containing 25 ng of penicillin G (0.04 U) over Brain Heart Infusion agar (Difco) (201) with supplements and were incubated anaerobically at 37°C for 12-14 hours. The cells were then suspended in 1 ml of sterile double distilled water and 150 µl of this suspension was mixed with 1 ml of 1% LMP agarose (GIBCO/BRL). The solidified agarose plugs were soaked overnight in 5 ml TE buffer (50 mM Tris, pH 8.0; 10 mM EDTA) at 4°C and then in achromopeptidase buffer (0.1 M Tris, pH 8.0; 0.1 M NaCl) for 30 min. at room temperature.

Embedded cells were lysed with achromopeptidase (2.5 µg/ml) and lysozyme (1 mg/ml) at 37°C for 30 min. and then with proteinase K (l mg/ml) in a solution containing 0.5 M EDTA, 0.5% lauryl sarcosinate (Sigma), and incubated at 50°C for 2-3 days (199). Slices of plugs were washed and embedded DNA was digested with *Xho*I. The selection of *Xho*I was based on the digestion of *P. magnus* ATCC 53516 with each of 8 restriction endonucleases, *Apa*I, *BamH*I, *Bgl*I, *Nhe*I, *Not*I, *Sal*I, *Sfi*I *and Xho*I; *Xho*I was chosen because it produced 14 well-separated fragments which met the requirements of using PFGE for the determination of relatedness (277). The resulting fragments were resolved on a 1% pulsed-field certified agarose (Bio-Rad) gel by PFGE at 14°C in 0.05 M TBE buffer (199) using the CHEF DRII apparatus (Bio-Rad). Program conditions for PFGE with linear ramping were as follows: initial switch time 1 S; final switch time 50 S, run time 15 h, to separate > 100 kb fragments; or, initial switch time 2 S, final switch time 15 S, run time 13.5 h, to separate 20-100 kb fragments. A lambda ladder (Promega Corp., Madison, WI.) was used as the molecular size marker. PFGE of *Xho*I digests were performed 5 times but only the best replicate was used for analysis.

**2.13 STATISTICAL ANALYSIS.**

Relatedness of isolates can be determined by comparing 2 isolates' macro restriction fragment size patterns using the Dice coefficient, *F*, which is defined as the ratio of twice the number of common bands found in each pair of restriction profiles to the sum total of macro restriction fragments in each profile (59, 196): $F = 2m_{xy}/m_X + m_y$ where $m_{xy}$ equals the number of common fragments in $m_x$ and $m_y$, $m_x$ equals the number of fragments in pattern x, and $m_y$ equals the number of fragments in pattern y (59). If two patterns are similar $F$=1.0, and if the two patterns are dissimilar $F$=0. Based on the $F$ values and the % similarity, a dendrogram was calculated using the unweighted pair group arithmetic average algorithm (the hierarchial cluster analysis) in the SPSS/PC+ package

(SPSS Inc.; Chicago, IL.). In this study, ribotyping results from *Hind*III and *Eco*RI digests, as well as from PFGE experiments were used singly or in combination to calculate *F* values. Designations of clusters of isolates were arbitrarily chosen at ≥70% similarity as the cut-off point. *F* values were re-confirmed using the the formula $F \approx P^t / (3\text{-}2P)$ (196) to show the degree of divergence between two isolates where *F* is the expected proportion of shared fragments and *P* is the probability that a restriction site has remained unaltered during time *t*. The clusters resulting from each set of *F* values obtained from ribotyping and/or PFGE were compared.

The unweighed pair-group method using arithmetic averages (UPGMA) is probably the most frequent method for dendrogram construction (144, 255). The UPGMA method has been shown to be an effective distance matrix method when distance estimates are subject to large stochastic errors (195). A limitation of the UPGMA method is that it assumes that the rates of evolution are equal among the different lineages so that an approximately linear relation exists between evolutionary distance and divergence time (194). Linear distance based on the number of nucleotide (or amino acid) substitutions can be used (144). The UPGMA method uses a sequential clustering algorithm, in which local topological relationships are identified in order of similarity, and the phylogenetic tree is built in a stepwise manner (144). In other words, from among all the operational taxonomic units (OTUs) available, the 2 OTUs that are most similar to each other are treated now as a new single OTU- called a composite OTU (255). Subsequently, from among the new group of OTUs, the pair with the greatest similarity is identified, and this process is continued until there are only 2 OTUs left (144). The distance between two composite OTUs is computed as the arithmetic mean of the distances between the constituent OTUs in each composite OTU (144).

PC SAS (SAS Institute, Cary, NC.) was used to calculate the Pearson's correlation which is

defined as:
$$r = \frac{\sum_{j=1}^{N} (X_i - \overline{X})(Y_i - \overline{Y})}{\sqrt{\sum_{j=1}^{N} (X_i - \overline{X})^2 (Y_i - \overline{Y})^2}}$$

$N$= number of cases; $X$= number of aminopeptidases

$Y$= % of gelatin hydrolysis; $\overline{X}$= mean of $X$; $\overline{Y}$= mean of $Y$

$i$= the $i$th value of $X$ or $Y$; $j$= the $j$th value of summation of cases.

The chi-square ($\chi^2$) test of homogeneity (125) was used to determine the association of gelatin hydrolysis with either species, the number of positive aminopeptidase reactions, combined positive aminopeptidase reactions, and the distribution of certain positive aminopeptidase reactions present among individual species.

The equality of proportions for more than 2 samples ($Z > \pm 1.96$) was used to determine if the presence of certain positive aminopeptidase reactions in each of the 5 *Peptostreptococcus* species was significant. The extended Tukey test (ANOVA, PC SAS) was used to determine if the mean percentage of positive aminopeptidase reactions produced by the 5 species were significant.

The Simpson's index of discrimination was performed on the 3 genotyping techniques (PCR-REA, ribotyping, and PFGE) to determine the discriminatory power of each method (110). The Simpson's index of discrimination is as follows:
$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j - 1)$$

$N$ = total number of strains in the sample population; $S$ = total number of types described; $n_j$ = number of strains belonging to the $j$th type.

# 3. RESULTS

## 3.1 IDENTIFICATION OF 5 *PEPTOSTREPTOCOCCUS* SPECIES BY AMINOPEPTIDASE PROFILES.

*Peptostreptococcus* isolates, grouped based on species and isolation site, were analyzed for their pre-formed enzyme profiles (Table 2). All *P. anaerobius* isolates had characteristic pre-formed enzyme profiles for αGLU and PRO (rapid ID32A index profile 0400020000) (Table 2). In addition, four of the 16 female genital tract *P. anaerobius* isolates hydrolyzed one of the 4 aminopeptidase synthetic substrates: ARG, LEU, ALA, or GLY (Table 2).

All *P. asaccharolyticus* isolates including the type strain ATCC 14963 had indole production and demonstrated ARG and HIS activity (Table 2). Eighty-eight percent of the isolates produced TYR and 72% produced LEU. Only one of 18 female genital tract isolates had a similar pre-formed enzyme profile as the type strain *P. asaccharolyticus* ATCC 14963.

All *P. magnus* isolates were asaccharolytic, and urease, arginine dihydrolase, nitrate reduction, and indole production negative. Nineteen of 24 *P. magnus* isolates, including the type strain, produced alkaline phosphatase (Table 2). All isolates were positive for ARG, LEU, and PYR, 95.8% of the isolates produced GLY, and 87.5% of the isolates produced LG and ALA. All isolates except for one female genital tract isolate had activity for GLY. One isolate from the female genital tract had activity for PRO, PHE, and GG. As a result, it was identified as *P. micros*.

As with *P. magnus* isolates, all 19 *P. micros* isolates were asaccharolytic, and urease, arginine dihydrolase, indole production, and nitrate reduction negative. The type strain *P. micros* ATCC 33270, all breast abscess and clinical isolates of unknown origin, and four of 11 female genital tract

**Table 2.** Pre-formed enzyme profile of 100 *Peptostreptococcus* strains and isolates and percentage of positive reactions.

| | No. with pre-formed enzymes[b,c] | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Peptostreptococcus* Species/strains[a] | ADH | αGLU | IND | PAL | ARG | PRO | LG | PHE | LEU | PYR | TYR | ALA | GLY | HIS | GG | SER |
| *P. anaerobius* | | | | | | | | | | | | | | | | |
| ATCC 27337 n=1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Female genital tract n=16 | 0 | 16 | 0 | 0 | 1 | 16 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| Unknown clinical origin n=3 | 0 | 3 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| % | 0 | 100 | 0 | 0 | 5 | 100 | 0 | 0 | 5 | 0 | 0 | 5 | 5 | 0 | 0 | 0 |
| *P. asaccharolyticus* | | | | | | | | | | | | | | | | |
| ATCC 14963 n=1 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| Female genital tract n=18 | 1 | 0 | 18 | 0 | 18 | 0 | 0 | 0 | 12 | 0 | 16 | 2 | 7 | 18 | 6 | 2 |
| Unknown clinical origin n=6 | 0 | 0 | 6 | 0 | 6 | 0 | 0 | 0 | 5 | 0 | 6 | 0 | 4 | 6 | 0 | 0 |
| % | 4 | 0 | 100 | 0 | 100 | 0 | 0 | 0 | 72 | 0 | 88 | 8 | 44 | 100 | 24 | 8 |
| *P. magnus* | | | | | | | | | | | | | | | | |
| ATCC 15794 n=1 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 |
| Female genital tract n=18 | 0 | 0 | 0 | 13 | 18 | 1 | 15 | 1 | 18 | 18 | 9 | 15 | 17 | 10 | 1 | 9 |
| Skin abscess n=2 | 0 | 0 | 0 | 2 | 2 | 0 | 2 | 0 | 2 | 2 | 0 | 2 | 2 | 0 | 0 | 0 |
| Unknown clinical origin n=3 | 0 | 0 | 0 | 3 | 3 | 0 | 3 | 1 | 3 | 3 | 1 | 3 | 3 | 3 | 0 | 3 |
| % | 0 | 0 | 0 | 79.2 | 100 | 4.2 | 87.5 | 8.3 | 100 | 100 | 45.8 | 87.5 | 95.8 | 58.3 | 4.2 | 54.2 |

**Table 2 continued.**

| *Peptostreptococcus* Species/strains[a] | No. with pre-formed enzymes[b,c] | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ADH | αGLU | IND | PAL | ARG | PRO | LG | PHE | LEU | PYR | TYR | ALA | GLY | HIS | GG | SER |
| *P. micros* | | | | | | | | | | | | | | | | |
| ATCC 14963 n=1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Breast abscess n=5 | 0 | 0 | 0 | 5 | 5 | 5 | 5 | 4 | 5 | 5 | 5 | 5 | 4 | 5 | 4 | 5 |
| Female genital tract n=11 | 0 | 0 | 0 | 4 | 11 | 3 | 9 | 3 | 11 | 10 | 6 | 9 | 10 | 9 | 3 | 10 |
| Unknown clinical origin n=2 | 0 | 0 | 0 | 2 | 2 | 2 | 1 | 2 | 2 | 2 | 2 | 2 | 1 | 2 | 2 | 2 |
| % | 0 | 0 | 0 | 63.2 | 100 | 57.9 | 84.2 | 52.6 | 100 | 94.7 | 73.7 | 89.5 | 84.2 | 89.5 | 52.6 | 94.7 |
| *P. prevotii*[d] | | | | | | | | | | | | | | | | |
| ATCC 9321 n=1 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 |
| Female genital tract n=5 | 1 | 0 | 0 | 3 | 5 | 0 | 3 | 0 | 5 | 3 | 3 | 2 | 4 | 3 | 2 | 3 |
| Unknown clinical origin n=6 | 2 | 0 | 0 | 1 | 6 | 1 | 3 | 1 | 4 | 2 | 2 | 5 | 6 | 6 | 1 | 5 |
| % | 25 | 8.3 | 0 | 33.3 | 100 | 8.3 | 50 | 16.7 | 83.3 | 50 | 50 | 66.7 | 91.7 | 83.3 | 25 | 75 |

[a] Identified by convential VPI method.

[b] Pre-formed enzymes of rapid ID32A; URE-urease; ADH-arginine dihydrolase; αGAL-alpha galactosidase; βGAL-beta galactosidase; βGP-beta galactosidase 6 phosphate; αGLU-alpha glucosidase; βGLU-beta glucosidase; αARA-alpha arabinosidase; βGUR-beta glucuronidase; βNAG-beta N-acetyl-glucosaminidase; MNE-mannose fermentation; RAF-raffinose fermentation; GDC-glutamic acid decarboxylase; αFUC-alpha fucosidase; NIT-nitrate reduction; IND-indole production; PAL-alkaline phosphatase; ARG-arginine arylamidase; PRO-proline arylamidase; LG-leucyl glycine arylamidase; PHE-phenylalanine arylamidase; LEU-leucine arylamidase; PYR-pyroglutamic acid arylamidase; TYR-tyrosine arylamidase; ALA-alanine arylamidase; GLY-glycine arylamidase; HIS-histidine arylamidase; GG-glutamyl glutamic acid arylamidase; and SER-serine arylamidase.

[c] All other 13 pre-formed enzyme reactions from rapid ID32A were negative except for *P. prevotii* isolates.

[d] For 12 *P. prevotii* isolates: 4 URE +; 3 ADH+; 1 αGAL+; 1 βGAL+; 5 αGLU+; 1 βGLU+; 4 βGUR +; 1 NIT+; and 4 PAL+.

isolates had strong activity for alkaline phosphatase production (Table 2). *P. micros* ATCC 33270 had activity for all 12 aminopeptidase reactions. Similar to *P. magnus*, all *P. micros* clinical isolates had activity for ARG and LEU, 94.7% of the isolates produced PYR and SER, 89.5% of the isolates produced ALA and HIS, and 84.2% of the isolates produced GLY and LG.

Four of the 12 *P. prevotii* isolates had urease activity, 3 produced arginine dihydrolase, 1 produced βGAL, 5 produced αGLU, 1 produced βGLU, 4 produced βGUR, 1 reduced nitrates, and 4 produced alkaline phosphatase (Table 3). All clinical isolates had activity for ARG, 91.7% of the isolates produced GLY, and 83.3% of the isolates produced LEU and HIS. (Table 2 and 3). Two *P. prevotii* clinical isolates of unknown origin (A129 and 83-125) and two female genital tract isolates (CS2620C and PID3401G) were identified as *P. magnus* by the kit had the following profile indices- 00000416405, 0000016407, 0000056700, and 0000056707. Whereas many of the enzymatic reactions for *P. prevotii* isolates were variable (very weak positive), this was not observed for most of the enzymatic reactions for the other *Peptostreptococcus* species in this study except for four aminopeptidase reactions of *P. anaerobius* isolates.

**3.2 SCREENING OF PROTEOLYTIC ACTIVITY BY GELATIN HYDROLYSIS.**

Forty-four of the 100 *Peptostreptococcus* isolates tested for aminopeptidase activity hydrolyzed gelatin weakly (Table 4). The types and number of positive aminopeptidase reactions in both gelatin positive and negative isolates of different *Peptostreptococcus* species are shown in Table 4.

The presence of certain positive aminopeptidase reactions in each of the 5 *Peptostreptococcus* species was found to be significant using the equality of proportions for more than 2 samples ($Z >$ 1,96) (Appendix C, Table 22). Statistical analysis using the $\chi^2$ analysis indicated there was a

**Table 3.** Pre-formed enzyme profile[a] of 12 *P. prevotii* obtained by rapid ID32A.

| STRAIN NO.[b] | URE | ADH | α GAL | β GAL | β GP | α GLU | β GLU | α ARA | β GUR | β NAG | MNE | RAF | GDC | α FUC | NIT | IND | PAL | ARG | PRO | LG | PHE | LEU | PYR | TYR | ALA | GLY | HIS | GG | SER |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ATCC 9321 | + | - | + | - | - | + | - | - | + | - | - | - | - | - | - | - | - | + | - | - | v | v | + | v | v | v | + | - | w+ |
| CS2620C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | v | - | + | v | - | v | v | w+ | - | w+ |
| PID676H | + | - | - | - | - | w+ | - | - | - | - | - | - | - | - | + | - | + | v | - | v | - | v | - | v | - | v | w+ | v | v |
| PID936J | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | + | - | - | - | + | - | - | - | w+ | + | - | - |
| PID990B | + | - | - | - | - | w+ | - | - | + | - | - | - | - | - | - | - | + | + | - | - | - | v | v | v | - | v | + | - | w+ |
| PID3401G | - | w+ | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | + | - | + | + | w+ | + | - | - | + | + |
| A129 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | v | - | + | v | - | v | v | w+ | - | w+ |
| 83-125 | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | v | - | + | - | - | v | v | + | - | v |
| 801478 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | v | + | v | - | v | w+ | w+ | v | v |
| 811556 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - | - | - | + | w+ | w+ | + | - | - |
| 831833 | + | + | - | + | - | + | + | - | + | - | - | - | - | - | - | - | + | + | - | - | - | + | - | + | + | + | + | - | w+ |
| AN2-253 | - | - | - | - | - | + | - | - | + | - | - | - | - | - | - | - | - | w+ | v | v | - | - | - | - | - | w+ | + | - | w+ |

[a] Refer to Table 2 for pre-formed enzyme abbreviations.

[b] Identification by conventional method.
+, positive; -, negative; w+, weak positive; v, variable (very weak positive).

[c] 100% positive for all 12 isolates.

significant association between gelatin hydrolysis and the *Peptostreptococcus* species ($n$=5) ($P$ < 0.001) (Appendix C, Table 23). *P. anaerobius* (2/20), *P. asaccharolyticus* (2/25), and *P. prevotii* (2/12) isolates usually did not hydrolyze gelatin whereas most *P. magnus* (23/24) and *P. micros* (15/19) isolates did hydrolyze gelatin (Table 5). The 5 species were classified into 3 groups using the extended Tukey test (ANOVA, PC SAS) ($P$ < 0.0001) based on the mean percentage of positive aminopeptidase reactions produced by each species. The groups with descending mean percentage of positive aminopeptidase reactions were: *P. magnus* and *P. micros* (group I); *P. asaccharolyticus* and *P. prevotii* (group II); and *P. anaerobius* (group III).

**3.3 PROTEOLYTIC ACTIVITY ON NONSPECIFIC AND GENERAL SUBSTRATES AND THE RELATIONSHIP OF AMINOPEPTIDASE PROFILES OF GELATIN POSITIVE AND NEGATIVE *PEPTOSTREPTOCOCCUS* ISOLATES.**

Four *P. magnus* isolates (Gifu 7651 and 7735, skin abscess isolates; and Gifu 7723 and 7654, ovarian abscess isolates) were chosen for a more intensive study to determine the substrate spectrum and level of proteolytic activity as compared to the reference strain, *P. gingivalis* W83. These *P. magnus* isolates were chosen because they were associated with soft tissue infections and the species has been noted for their proteolytic activities (28, 127-128). Even after concentrating *P. magnus* cells 10 times more than the reference strain, only weak proteolytic activity was observed for gelatin, fibrinogen, BSA, casein, and autoclaved collagen and no proteolytic activity was observed for the other substrates tested (Table 6).

The aminopeptidase reaction profiles for 44 gelatin positive and 56 gelatin negative *Peptostreptococcus* isolates are shown in Table 4. Linear regression analysis showed a significant correlation between the percentage of gelatin hydrolyzing isolates of *Peptostreptococcus* species and

**Table 4.** Distribution of aminopeptidases in profiles of both gelatin positive and gelatin negative *Peptostreptococcus* isolates from 5 species.

| Species | Gelatin[a] Hydrolysis | Aminopeptidase profile[a,b] | | | | | | | | | | | | No. Isolates |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ARG | PRO | LG | PHE | LEU | PYR | TYR | ALA | GLY | HIS | GG | SER | |
| *P. anaerobius* | +[c] | - | Pro | - | - | - | - | - | - | - | - | - | - | 2 |
| | - | - | Pro | - | - | - | - | - | - | - | - | - | - | 14 |
| | | Arg | Pro | - | - | - | - | - | - | - | - | - | - | 1 |
| | | - | Pro | - | - | Leu | - | - | - | - | - | - | - | 1 |
| | | - | Pro | - | - | - | - | - | Ala | - | - | - | - | 1 |
| | | - | Pro | - | - | - | - | - | - | Gly | - | - | - | 1 |
| | | | | | | | | | | | | | Total | 20 |
| *P. asaccharolyticus* | +[c] | Arg | - | - | - | Leu | - | - | - | - | His | - | - | 1 |
| | | Arg | - | - | - | Leu | - | Tyr | - | - | His | GG | - | 1 |
| | - | Arg | - | - | - | Leu | - | - | - | - | His | - | - | 2 |
| | | Arg | - | - | - | - | - | Tyr | - | - | His | - | - | 5 |
| | | Arg | - | - | - | Leu | - | Tyr | - | - | His | - | - | 3 |
| | | Arg | - | - | - | - | - | Tyr | - | - | His | - | Ser | 1 |
| | | Arg | - | - | - | Leu | - | Tyr | - | Gly | His | - | - | 7 |
| | | Arg | - | - | - | Leu | - | Tyr | - | - | His | GG | - | 1 |
| | | Arg | - | - | - | Leu | - | Tyr | - | Gly | His | GG | - | 2 |
| | | Arg | - | - | - | Leu | - | Tyr | Ala | Gly | His | GG | - | 1 |
| | | Arg | - | - | - | - | - | Tyr | Ala | Gly | His | GG | Ser | 1 |
| | | | | | | | | | | | | | Total | 25 |

**Table 4 continued.**

| Species | Gelatin[a] Hydrolysis | Aminopeptidase profile[a,b] | | | | | | | | | | | | No. Isolates |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ARG | PRO | LG | PHE | LEU | PYR | TYR | ALA | GLY | HIS | GG | SER | |
| *P. magnus* | + | Arg | - | LG | - | Leu | Pyr | - | - | - | - | - | - | 1 |
| | | Arg | - | - | - | Leu | Pyr | - | - | Gly | - | - | - | 2 |
| | | Arg | - | - | - | Leu | Pyr | Tyr | Ala | Gly | - | - | - | 1 |
| | | Arg | - | LG | - | Leu | Pyr | - | Ala | Gly | - | - | - | 6 |
| | | Arg | - | LG | - | Leu | Pyr | - | Ala | Gly | His | - | - | 1 |
| | | Arg | - | LG | - | Leu | Pyr | - | Ala | Gly | His | - | Ser | 3 |
| | [c] | Arg | - | LG | - | Leu | Pyr | Tyr | Ala | Gly | His | - | Ser | 8 |
| | | Arg | - | LG | Phe | Leu | Pyr | Tyr | Ala | Gly | His | - | Ser | 1 |
| | - | Arg | Pro | LG | Phe | Leu | Pyr | Tyr | Ala | Gly | His | GG | Ser | 1 |
| | | | | | | | | | | | | | Total | 24 |
| *P. micros* | + | Arg | - | - | - | Leu | Pyr | - | Ala | Gly | His | - | Ser | 1 |
| | | Arg | Pro | LG | Phe | Leu | Pyr | - | - | - | - | GG | - | 1 |
| | | Arg | - | LG | - | Leu | Pyr | - | - | Gly | His | - | Ser | 1 |
| | | Arg | - | LG | - | Leu | Pyr | Tyr | Ala | Gly | - | - | Ser | 1 |
| | | Arg | - | LG | - | Leu | Pyr | - | Ala | Gly | His | - | Ser | 2 |
| | | Arg | - | LG | - | Leu | Pyr | Tyr | Ala | Gly | His | - | Ser | 2 |
| | | Arg | Pro | LG | - | Leu | Pyr | Tyr | Ala | - | His | GG | Ser | 1 |
| | | Arg | Pro | LG | Phe | Leu | Pyr | Tyr | Ala | Gly | His | - | Ser | 1 |

**Table 4 continued.**

| Species | Gelatin[a] Hydrolysis | Aminopeptidase profile[a,b] | | | | | | | | | | | | No. Isolates |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ARG | PRO | LG | PHE | LEU | PYR | TYR | ALA | GLY | HIS | GG | SER | |
| | [c] | Arg | Pro | LG | Phe | Leu | Pyr | Tyr | Ala | Gly | His | GG | Ser | 5 |
| | - | Arg | - | LG | - | Leu | Pyr | Tyr | Ala | Gly | His | - | Ser | 1 |
| | | Arg | Pro | - | Phe | Leu | - | Tyr | Ala | Gly | His | GG | Ser | 1 |
| | | Arg | Pro | - | Phe | Leu | Pyr | Tyr | Ala | - | His | GG | Ser | 1 |
| | | Arg | Pro | LG | Phe | Leu | Pyr | Tyr | Ala | Gly | His | GG | Ser | 1 |
| | | | | | | | | | | | | | Total | 19 |
| *P. prevotii* | + | Arg | - | LG | - | Leu | Pyr | - | Ala | Gly | - | - | - | 1 |
| | | Arg | Pro | - | - | Leu | - | Tyr | Ala | Gly | His | - | Ser | 1 |
| | - | Arg | - | - | - | Leu | - | - | - | Gly | His | - | - | 1 |
| | | Arg | - | - | - | - | - | Tyr | Ala | Gly | His | - | - | 1 |
| | | Arg | - | LG | - | - | - | - | - | Gly | His | - | Ser | 1 |
| | | Arg | - | LG | - | Leu | - | - | Ala | Gly | His | - | Ser | 1 |
| | | Arg | - | - | - | Leu | Pyr | Tyr | - | Gly | His | - | Ser | 1 |
| | | Arg | - | LG | - | Leu | Pyr | Tyr | Ala | - | - | GG | Ser | 1 |
| | | Arg | - | LG | - | Leu | - | Try | - | Gly | His | GG | Ser | 1 |
| | | Arg | - | LG | - | Leu | Pyr | - | Ala | Gly | His | - | Ser | 1 |
| | [c] | Arg | - | - | Phe | Leu | Pyr | Tyr | Ala | Gly | His | - | Ser | 1 |
| | | Arg | - | - | Phe | Leu | Pyr | - | Ala | Gly | His | GG | Ser | 1 |
| | | | | | | | | | | | | | Total | 12 |

[a] Chi-square analysis between gelatin hydrolysis and the number of aminopeptidase positive reactions. $\chi^2 > 10.83$; $P < 0.001$ (See Appendix C, Table 25).

[b] ARG-arginine; PRO-proline; LG-leucyl glycine; PHE-phenylalanine; LEU-leucine; PYR-pyroglutamic acid; TYR-tyrosine; ALA-alanine; GLY-glycine; HIS-Histidine; GG-glutamyl glutamic acid; and SER-serine.

[c] ATCC type strain.

**Table 5.** Relationship between the number of aminopeptidase substrates hydrolyzed by 44 *Peptostreptococcus* isolates and gelatin hydrolysis.[a]

| Species[c] | Number of aminopeptidase reactions[b] produced by isolates hydrolyzing gelatin | | | | | | | | | | | | % hydrolyzing gelatin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. of strains | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | |
| *P. anaerobius* | 2/20 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10% |
| *P. asaccharolyticus* | 2/25 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8% |
| *P. magnus* | 23/24 | 0 | 0 | 0 | 3 | 0 | 7 | 1 | 3 | 8 | 1 | 0 | 0 | 95.8% |
| *P. micros* | 15/19 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 3 | 2 | 1 | 1 | 5 | 78.9% |
| *P. prevotii* | 2/12 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 16.7% |

[a] Chi-square analysis between gelatin hydrolysis and *Peptostreptococcus* species: $\chi^2 > 18.47$; $P < 0.001$ (see Appendix B, Table 23).

[b] Positive aminopeptidase reactions detected using the Rapid ID32A kit (See Table 4).

[c] As speciated by the VPI method.

the number of positive aminopeptidase reactions produced by *Peptostreptococcus* isolates ($r$=0.80; $P$=0.0019) (Appendix C, Table 24). The % of gelatin hydrolysis ($y$-axis) versus the number of positive aminopeptidase reactions for 100 *Peptostreptococcus* isolates ($x$-axis) has been plotted (Figure 2). The 100 isolates were then arbitrarily divided into 2 groups: group 1 (47 isolates) produced 1 to 5 positive aminopeptidase reactions and group 2 (53 isolates) produced 6 to 12 positive aminopeptidase reactions. $\chi^2$ analysis on the rearranged data showed a significant association ($P < 0.001$) between the diversity of positive aminopeptidase reactions produced and gelatin hydrolysis (Appendix C, Table 25). Seventy percent of group 2 isolates hydrolyzed gelatin and 94.6% of these were *P. magnus* ($n$=19) and *P. micros* ($n$=15) isolates. Amongst group 1 isolates, 14.9% hydrolyzed gelatin and 44 of the 47 isolates were *P. anaerobius* ($n$=20), *P. asaccharolyticus* ($n$=21), and *P. prevotii* ($n$=3). In summary, statistical analysis showed that species with more proteolytic isolates, *P. magnus* and *P. micros*, had more positive aminopeptidase reactions (4 to 12) than the generally non-proteolytic species, *P. anaerobius* (1 to 2), *P. asaccharolyticus* (3 to 7), and *P. prevotii* (4 to 9).

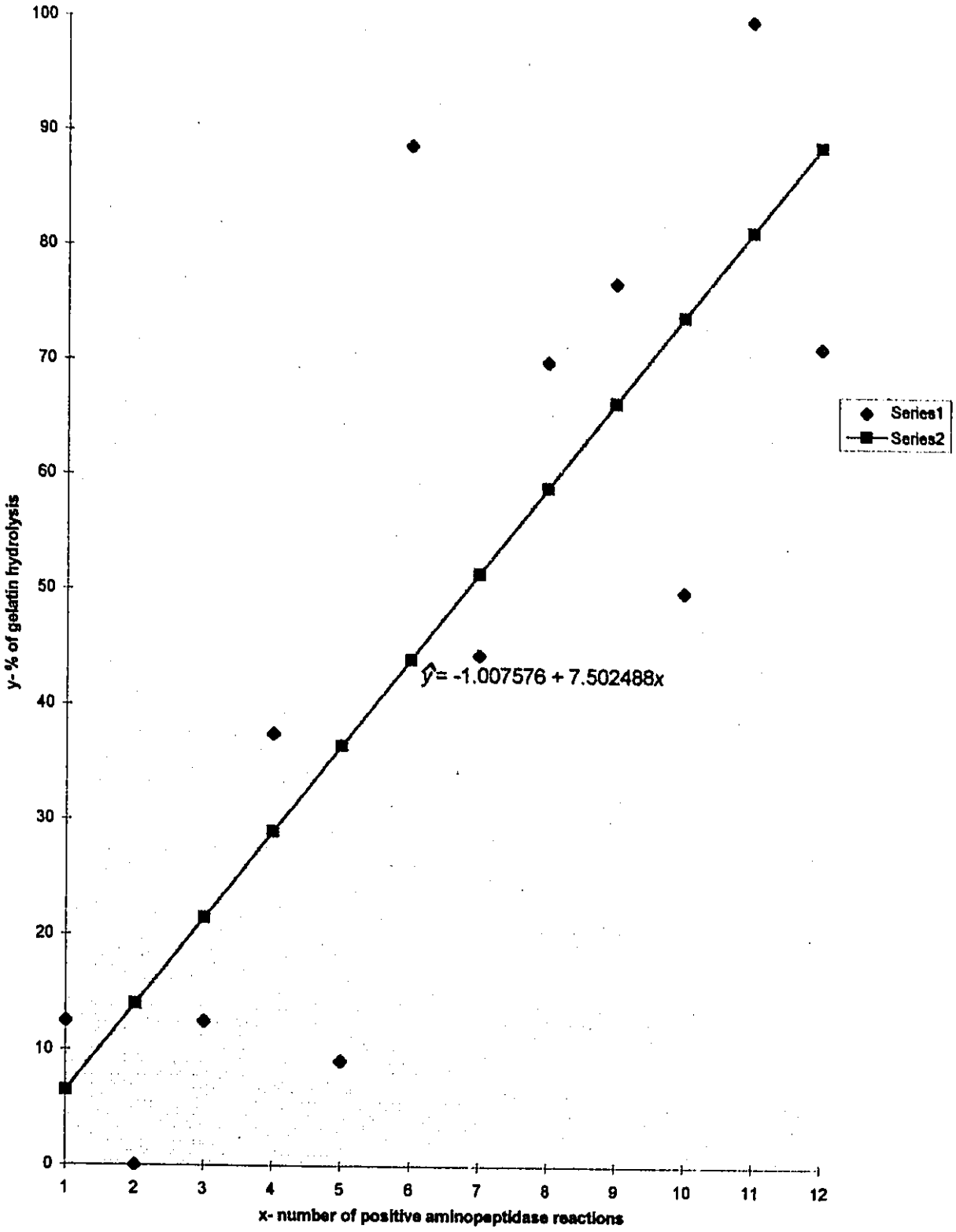
Combining results from all species, $\chi^2$ analysis showed there was an association between gelatin hydrolysis and positive aminopeptidase reactions for all 5 species of *Peptostreptococcus* ($P < 0.001$). Furthermore, $\chi^2$ analysis showed there was a significant association between gelatin hydrolysis and the distrtibution of certain positive aminopeptidase reactions present among individual species (Appendix C, Table 26). These included ARG, LG, LEU, PYR, TYR, ALA, GLY, HIS, and SER (all were $P < 0.001$) (Table 1). The variability of gelatin hydrolysis among isolates with the same aminopeptidase profile (e.g. *P. anaerobius* isolates with only PRO can be both gelatin positive or negative) could be due to factors like the quantity, and/or activity of positive aminopeptidase

**Table 6.** Semi-quantitative analysis of 4 *Peptostreptococcus magnus* isolates as compared to *Porphyromonas gingivalis* W83 on nonspecific and general substrates

| Substrate | Proteolytic activity of[a,b] | | | | |
|---|---|---|---|---|---|
| | *P. gingivalis* W83 | *P. magnus* Gifu 7651 | *P. magnus* Gifu 7723 | *P. magnus* Gifu 7735 | *P. magnus* Gifu 7654 |
| Gelatin | 100 | 22 | 26 | 13 | 30 |
| Fibrinogen | 100 | 14 | 15 | 11 | 15 |
| BSA | 100 | 15 | 17 | 8 | 17 |
| Casein | 100 | 15 | 16 | 9 | 15 |
| Azocoll | 100 | 0 | 0 | 0 | 0 |
| Collagen | 100 | 0 | 0 | 0 | 0 |
| Collagen (autoclaved) | 100 | 28 | 37 | 15 | 30 |
| Benzoyl-arginine-*p*-nitroanilide | 100 | 0 | 0 | 0 | 0 |
| Glycyl-proline-*p*-nitroanilide | 100 | 0 | 0 | 0 | 0 |
| Casein yellow | 100 | 0 | 0 | 0 | 0 |
| Azocasein | 100 | 0 | 0 | 0 | 0 |

[a] The values (%) obtained are relative to the reference strain *P. gingivalis* W83.
[b] Peptostreptococcal cells were 10 times more concentrated than *P. gingivalis* W83.

**Figure 2.** Linear regression analysis of the % gelatin hydrolysis (y-axis) vs the number of positive aminopeptidase reactions for 100 *Peptostreptococcus* isolates (x-axis). Series 1 is the % of isolates that hydrolyze gelatin with x number of positive aminopeptidase reactions (y-values) and series 2 is based on $\hat{y}$ values ($y$-values predicted by a straight line for a value of $x$).


100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
y- % of gelatin hydrolysis
x- number of positive aminopeptidase reactions
ŷ= -1.007576 + 7.502488x
Series1
Series2

reactions besides the number and types of positive aminopeptidase reactions.

### 3.4 SENSITIVITY AND SPECIFICITY OF RAPID ID32A.

In the evaluation of the rapid ID32A kit, the overall sensitivity and specificity in the identification of the 5 *Peptostreptococcus* species by the rapid ID32A kit as compared to the VPI method as the "gold" standard was 93% and 80%, respectively (Table 7). All *P. anaerobius* ($n$=20) and *P. asaccharolyticus* ($n$=25) isolates were correctly identified, as compared to the conventional method, with 100% sensitivity and 100% specificity (Table 7). With *P. magnus* isolates ($n$=24), the specificity was 95.8% with only one isolate being misidentified as *P. micros*. The rapid ID32A kit was 57.9% specific for the identification of *P. micros* isolates; eight of 19 isolates were misidentified as *P. magnus*. Only one of 12 *P. prevotii* isolates, the type strain ATCC 9321, was identified correctly using the VPI speciation criteria by the rapid ID32A kit thereby producing a very low specificity of 8.3% (Table 7). Four *P. prevotii* isolates were identified as *P. magnus*. The rest were unidentifiable, thus the sensitivity (41.7%) and specificity (8.3%) of the kit for the identification of *P. prevotii* was lower compared to the identification of the other four *Peptostreptococcus* species.

### 3.5 DETECTION AND SUBTYPING OF PROTEIN L IN 32 *P. MAGNUS* ISOLATES BY PCR-REA.

Protein L has been correlated to *P. magnus* isolates from BV patients and may serve as a virulence factor (116). To determine the commonality of protein L gene sequences in *P. magnus*, thirty-two isolates from different countries and isolation sites were screened by PCR with primers LP1 and LP4. Only three isolates from Sweden which were previously shown to contain protein L (ATCC 53516, 564, and 624) produced the expected 1.1 kb amplicon (117), which was confirmed by hybridization to the LP5 probe. The amplicons produced identical restriction endonuclease profiles

**Table 7.** Sensitivity and specificity of rapid ID32A for identifying five *Peptostreptococcus* species compared to the "gold" standard VPI method.

| Species[a] | No. | *P. anaerobius* | *P. asaccharolyticus* | *P. magnus* | *P. micros* | *P. prevotii* | No ID | % Sensitivity[b] | % Specificity[c] |
|---|---|---|---|---|---|---|---|---|---|
| *P. anaerobius* | 20 | 20 | | | | | | 100.0 | 100.0 |
| *P. asaccharolyticus* | 25 | | 25 | | | | | 100.0 | 100.0 |
| *P. magnus* | 24 | | | 23 | 1 | | | 100.0 | 95.8 |
| *P. micros* | 19 | | | 8 | 11 | | | 100.0 | 57.9 |
| *P. prevotii* | 12 | | | 4 | | 1 | 7 | 41.7 | 8.3 |
| Total | 100 | | | | | | | 93.0 | 80.0 |

[a] Identification obtained by VPI conventional method including GLC.
[b] Sensitivity: (# of *Peptostreptococcus* isolates with positive test/ # of *Peptostreptococcus* isolates tested based on VPI) X 100%.
[c] Specificity: (# of *Peptostreptococcus* isolates correctly identified/ # of *Peptostreptococcus* isolates tested) X 100%.

after digestion with: *Acc*I, *Eco*RI, *Hin*dIII, *Pst*I, and *Taq*I. The expected fragment sizes obtained from PCR-REA of the 1.1 kb protein L amplicons for the three isolates are shown in Table 8. The same three *P. magnus* isolates produced identical amplicons (approximately 650 bp) when degenerate protein L-based primers, DLP1 and DLP4, were used. REA profiles showed that the 652 bp amplicon was a truncated version of the 1.1 kb amplicon (Figure 1 and 3). The expected fragment sizes obtained from PCR-REA of the 652 bp degenerate protein L amplicon for the three isolates are shown in Table 9. All other isolates did not produce amplicons using either sets of primers.

Fourteen isolates, including $L^-A^+$ isolates KA, BL 1116, BL 1730, BL 2771, BL 3410, BL 3431, A95 and ATCC 15794, the 3 $L^+A^-$ isolates (ATCC 53516, 564 and 624), and 3 $L^-A^-$ isolates (595, AN2-970 and 791273) were selected for subtyping using the molecular analyses to determine the genetic make-up of these phenotypes as described below.

**3.6 PCR-REA OF 16S rRNA SEQUENCES.**

Amplification of 16S rRNA sequences using primers fD1 and rD1 produced a 1.5 kb amplicon in all fourteen isolates. Each of these amplicons produced similar digestion patterns when digested with one of *Alu*I, *Hin*fI, *Hpa*II, or *Sty*I (Figure 4 and Table 10). *Hin*fI digestion of these amplicons resulted in two fragments at 1.1 kb and 0.4 kb. *Alu*I digestion of these amplicons resulted in five fragments at 0.53 kb, 0.27 kb, 0.25 kb, 0.24 kb, and 0.20 kb. *Hpa*II digestion of these amplicons resulted in five fragments at 0.89 kb, 0.19 kb, 0.16 kb, 0.14 kb, and 0.13 kb. *Sty*I digestion of these amplicons resulted in three fragments at 1.06 kb, 0.28 kb, and 0.12 kb. However, *Taq*I and *Sau*3AI digestion of these amplicons each produced two patterns (Table 10); the three $L^+A^-$ isolates and 2 of 3 $L^-A^-$ isolates (595 and AN2-970) had five similar *Taq*I fragments (0.53 kb, 0.48 kb, 0.37 kb, 0.09 kb, and 0.07 kb; Figure 5, pattern 2). The eight $L^-A^+$ isolates and isolate 791273 ($L^-A^-$) had

**Table 8.** Expected[a] DNA fragment sizes of restriction endonuclease analysis of the 1,152 bp protein L amplicon from *P. magnus* ATCC 53516.

| Restriction endonuclease | Fragment size (kb) |
|---|---|
| *Acc*I | 0.64, 0.28, 0.22 |
| *Eco*RI | 0.36, 0.33, 0.22 |
| *Hin*dIII | 0.76, 0.37 |
| *Pst*I | 0.80, 0.33 |
| *Taq*I | 1.0, 0.073, 0.054 |

[a] Based on reported DNA sequence (117).

**Figure 3.** Restriction endonuclease profiles of the 652 bp degenerate protein L amplicon. Digestion with *Acc*I resolved into a 0.64 kb band (Lane 1), *Eco*RI resolved into 0.36 kb and 0.23 kb bands (Lane 2), *Hin*dIII resolved into 0.38 kb and 0.28 kb bands (Lane 3), *Pst*I resolved into 0.34 kb and 0.31 kb bands (Lane 4), or *Taq*I reolved into a 0.48 kb band (Lane 5) of *P. magnus* ATCC 53516. Specific digests produced similar profiles for all 3 protein L-containing *P. magnus* isolates. Amplicons were resolved on 3% MetaPhor™ agarose. Marker: AmpliSize standard (2 kb to 50 bp; Bio-Rad).

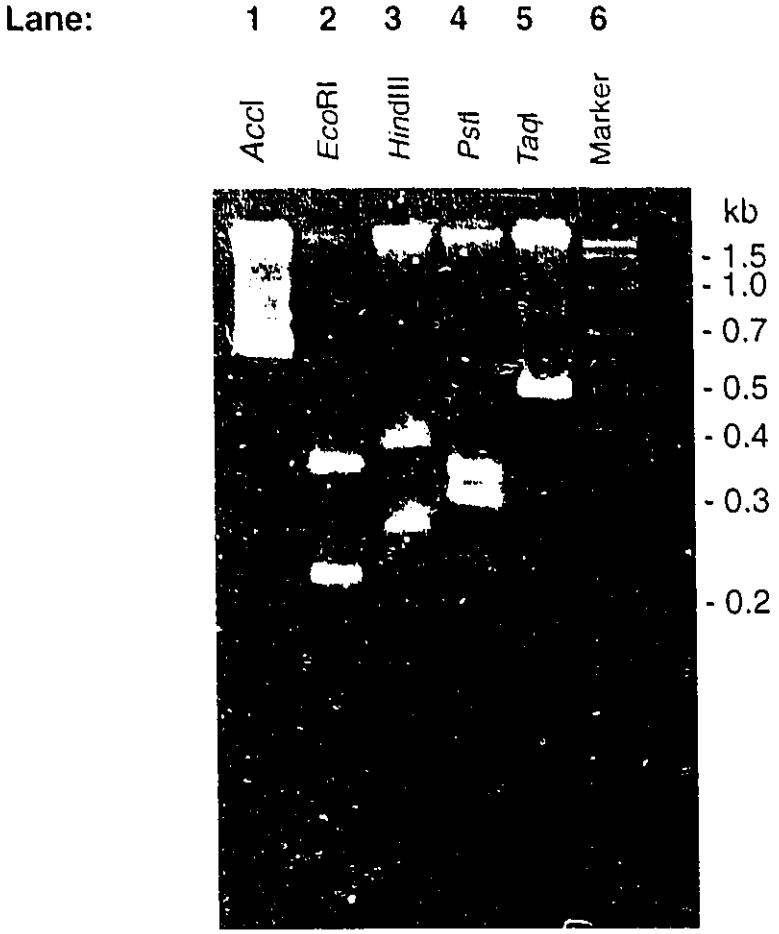

Lane:
1
2
3
4
5
6
AccI
EcoRI
HindIII
PstI
TaqI
Marker
kb
- 1.5
- 1.0
- 0.7
- 0.5
- 0.4
- 0.3
- 0.2

**Table 9.** Expected[a] DNA fragment sizes of restriction endonuclease analysis of the degenerate 652 bp amplicon of protein L from ATCC 53516.

| Restriction endonuclease | Fragment size (kb) |
|---|---|
| *Acc*I | 0.64, 0.012 |
| *Eco*RI | 0.36, 0.23, 0.059 |
| *Hin*dIII | 0.38, 0.28 |
| *Pst*I | 0.34, 0.31 |
| *Taq*I | 0.48, 0.128, 0.054 |

[a] Based on reported DNA sequence (117).

**Figure 4.** Restriction endonuclease analysis of the 1.5 kb amplicon of the 16S rRNA gene from one representative of the 14 *P. magnus* isolates after digestion with *Hin*fI (Figure A, Lane 2), *Alu*I (Figure B, Lane 2), *Hpa*II (Figure C, Lane 2), or *Sty*I (Figure C, Lane 3). Specific digests produced similar profiles for all 14 isolates. The DNA fragments at 0.48 kb and 0.85 kb are partials for the *Alu*I digest. DNA fragments at 0.62 kb and 0.26 kb are partials for the *Hpa*II digest. Amplicons were resolved on 3% MetaPhor™ agarose. Marker: AmpliSize marker (2 kb to 50 bp; Bio-Rad).

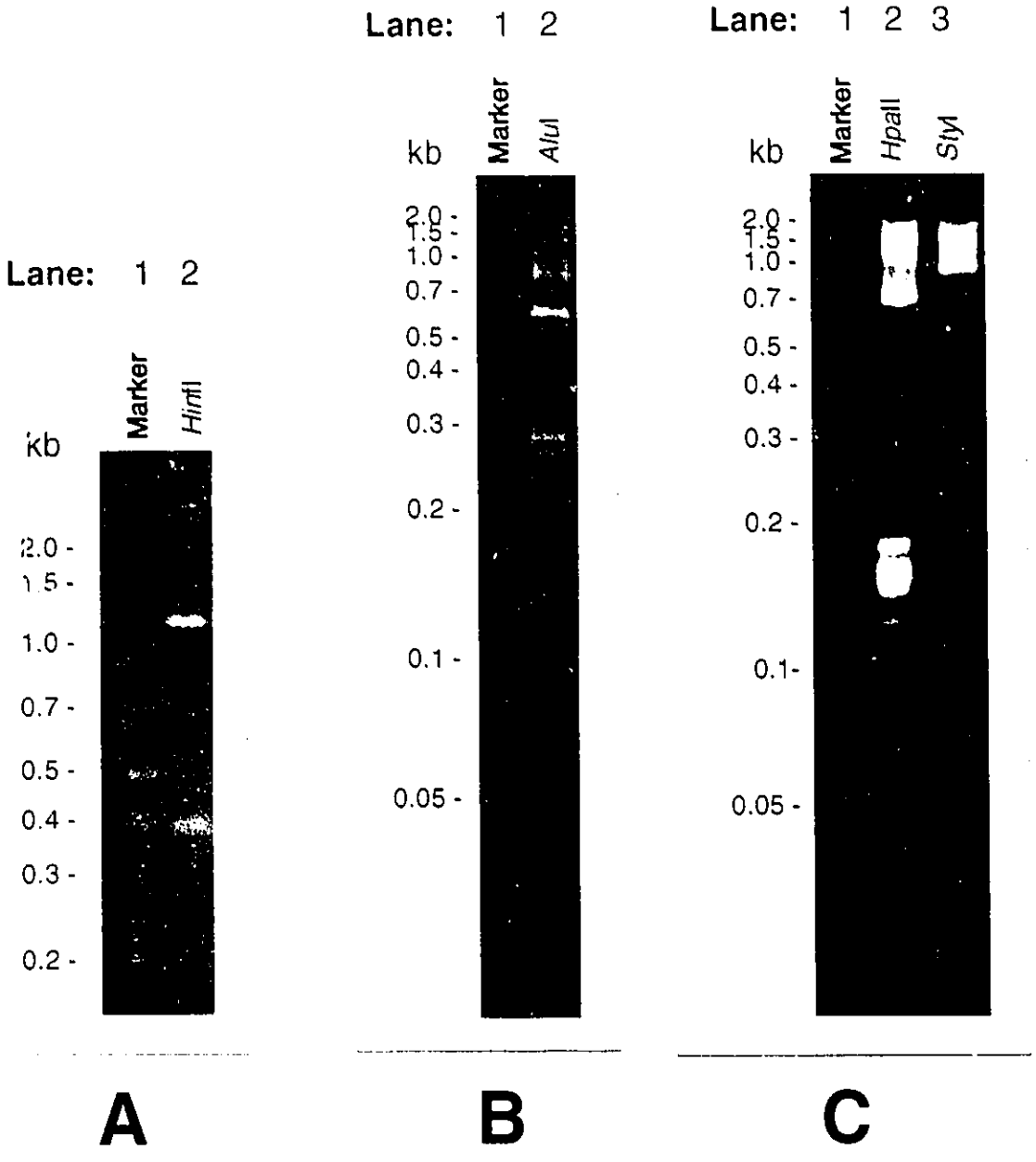

Lane: 1 2
Marker
HinfI
kb
2.0
1.5
1.0
0.7
0.5
0.4
0.3
0.2
A
Lane: 1 2
Marker
AluI
kb
2.0
1.5
1.0
0.7
0.5
0.4
0.3
0.2
0.1
0.05
B
Lane: 1 2 3
Marker
HpaII
StyI
kb
2.0
1.5
1.0
0.7
0.5
0.4
0.3
0.2
0.1
0.05
C

**Table 10.** Restriction endonuclease analysis profiles of the 1.5 kb 16S rRNA gene from up to 14 *P. magnus* isolates resolved on 4% AmpliSize or 3% MetaPhor™ agarose gels[a].

| Restriction endonuclease | | Fragment size[b, c] (kb) |
|---|---|---|
| *Alu*I | | 0.53 ± 0.02, 0.27 ± 0.02, 0.25 ± 0.01, 0.24 ± 0.01, 0.20 ± 0.01 |
| *Hin*fI | | 1.10, 0.40 |
| *Hpa*II | | 0.89 ± 0.06, 0.19, 0.16, 0.14 ± 0.02, 0.13 |
| *Sty*I | | 1.06 ± 0.06, 0.28 ± 0.01, 0.12 |
| *Sau*3AI | Pattern i | 0.74 ± 0.007, 0.30 ± 0.007, 0.27 ± 0.007, 0.16 ± 0.007[d] |
| | Pattern ii | 0.74 ± 0.007, 0.30 ± 0.007, 0.27 ± 0.007, 0.16 ± 0.007, 0.15[e] |
| *Taq*I | Pattern 1 | 0.53 ± 0.02, 0.37 ± 0.02, 0.32 ± 0.008, 0.19 ± 0.02, 0.09 ± 0.008, 0.07 ± 0.006[f] |
| | Pattern 2 | 0.53 ± 0.01, 0.48 ± 0.02, 0.37 ± 0.02, 0.09 ± 0.01, 0.07 ± 0.007[g] |

[a] Weak bands considered to be partial digests not included.

[b] Average values from 5 replicates of the 14 *P. magnus* isolates.

[c] Standard deviation $(S_D)=\sqrt{\sum_{j=1}^{N}(X-\overline{X})^2/n\text{-}1}$; $X$- size of DNA fragment; $\overline{X}$- mean of $X$; $n$- number of cases; $i$ - the $i$th value of $X$; $j$ - the $j$th value of summation of cases.

[d] All 14 *P. magnus* isolates except ATCC 15794, A95 and 791273.

[e] ATCC 15794, A95 and 791273.

[f] All 14 *P. magnus* isolates except 595, ATCC 53516, 564, 624 and AN2-970.

[g] 595, ATCC 53516, 564, 624 and AN2-970.

six *Taq*I restriction fragments (Figure 5, pattern 1) which were similar to pattern 2, except that the 0.48 kb fragment was absent but has been resolved probably into 0.32 kb and 0.19 kb fragments (Table 10). The 0.09 kb and 0.07 kb *Taq*I fragments were not reproducible because the bands were faint (Figure 5). With the *Sau*3AI digestion, two patterns were also seen (Table 10): eleven of 14 *P. magnus* isolates had one pattern (0.74 kb, 0.30 kb, 0.27 kb, and 0.16 kb bands) (Figure 6, pattern i); however, type strain ATCC 15794 ($L^-$ $A^+$), A95 ($L^-$ $A^+$) and 791273 ($L^-$ $A^-$) had an extra *Sau*3AI band of 0.15 kb (Figure 6, pattern ii). The PCR-REA of the 16S rRNA amplicons were reproducible giving the same patterns and band sizes in five different replicates. REA profiles of the 16S rRNA amplicon for the 14 *P. magnus* isolates were similar after digestion with one of *Alu*I, *Hin*fI, *Hpa*II, or *Sty*I. However, with the restriction endonucleases that have a 4 base pair recognition site for digestion (*Sau*3AI and *Taq*I), the 14 *P. magnus* isolates produced two banding group patterns.

**3.7 RIBOTYPING.**

The fourteen *P. magnus* isolates were differentiated into nine ribotype patterns following digestion of chromosomal DNA with *Hin*dIII; sizes ranged from 1.4-9.8 kb designated a to i (Figure 7). The fragment sizes obtained for the *Hin*dIII-based ribotypes are listed (Table 11). The three $L^+$ $A^-$ isolates were typed into two patterns, d and e (Table 14) which contained five common bands: strain ATCC 53516 (Figure 7) comprised pattern d while isolates 564 and 624 belonged to pattern e (Figure 7). The eight $L^-$ $A^+$ isolates were grouped into four patterns (Table 14) with Swedish isolates KA, BL 1116, and BL 3431 belonging to pattern a (Figure 7), Swedish isolates BL 1730, BL 2771, and BL 3410 belonging to pattern b and type strain ATCC 15794 and Canadian isolate A95 having distinctive patterns f and g. Both patterns a and b contained seven bands but four of these bands differed in size between the two. The three $L^-$ $A^-$ isolates 595, AN2-970 and 791273 each had

**Figure 5.** *TaqI* digestion of the 1.5 kb 16S rRNA PCR amplicon from 14 *P. magnus* isolates on 3% MetaPhor™ agarose gel. *P. magnus* isolates fell into banding pattern 1 or 2. Pattern 1: KA, BL 1116, BL 1730, BL 2771, BL 3410, BL 3431, ATCC 15794, and A95 ($L^- A^+$); and 791273 ($L^- A^-$). Pattern 2: ATCC 53516, 564, and 624 ($L^+ A^-$) and 595 and AN2-970 ($L^- A^-$). The 0.09 kb and 0.07 kb fragments were not visible in this figure. Marker: AmpliSize marker (2 kb to 50 bp; Bio-Rad).

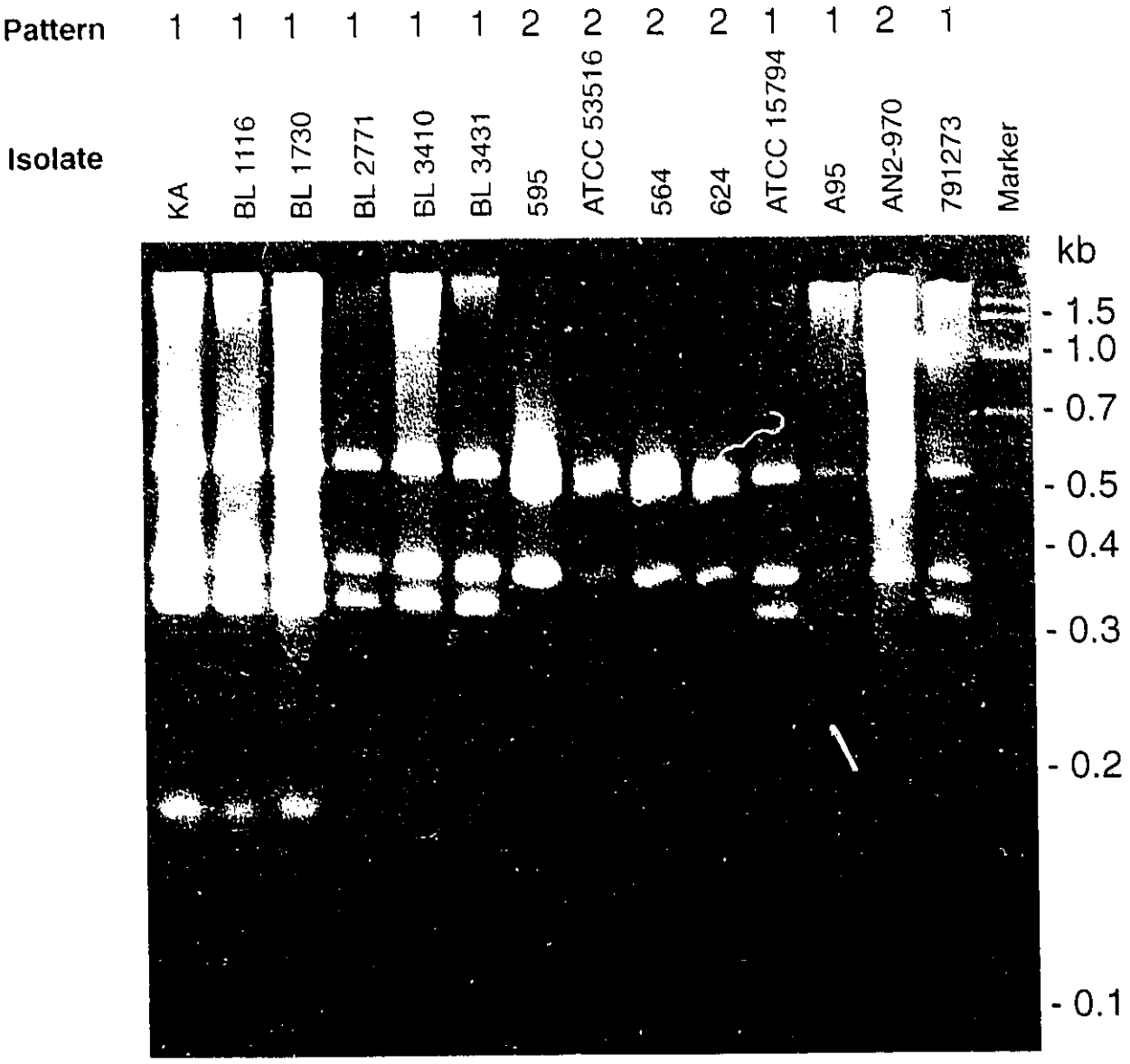

Pattern
1 1 1 1 1 1 2 2 2 2 1 1 2 1
Isolate
KA
BL 1116
BL 1730
BL 2771
BL 3410
BL 3431
595
ATCC 53516
564
624
ATCC 15794
A95
AN2-970
791273
Marker
kb
- 1.5
- 1.0
- 0.7
- 0.5
- 0.4
- 0.3
- 0.2
- 0.1

**Figure 6.** *Sau*3AI digestion of the 1.5 kb 16S rRNA amplicon from 14 *P. magnus* isolates. Amplicons were resolved on 3% MetaPhor™ agarose. Pattern i: KA, BL 1116, BL 1730, BL 2771, BL 3410, and BL 3431 ($L^- A^+$); ATCC 53516, 564, and 624 ($L^+ A^-$); 595 and AN2-970 ($L^- A^-$). Pattern ii: ATCC 15794 and A95 ($L^- A^+$); and 791273 ($L^- A^-$). The DNA fragments at 0.56 kb (lanes 9-13, 15), 0.51 kb (lane 13) and 0.22 kb (lane 13) are partials. Marker: AmpliSize marker (2 kb-50 bp; Bio-Rad).

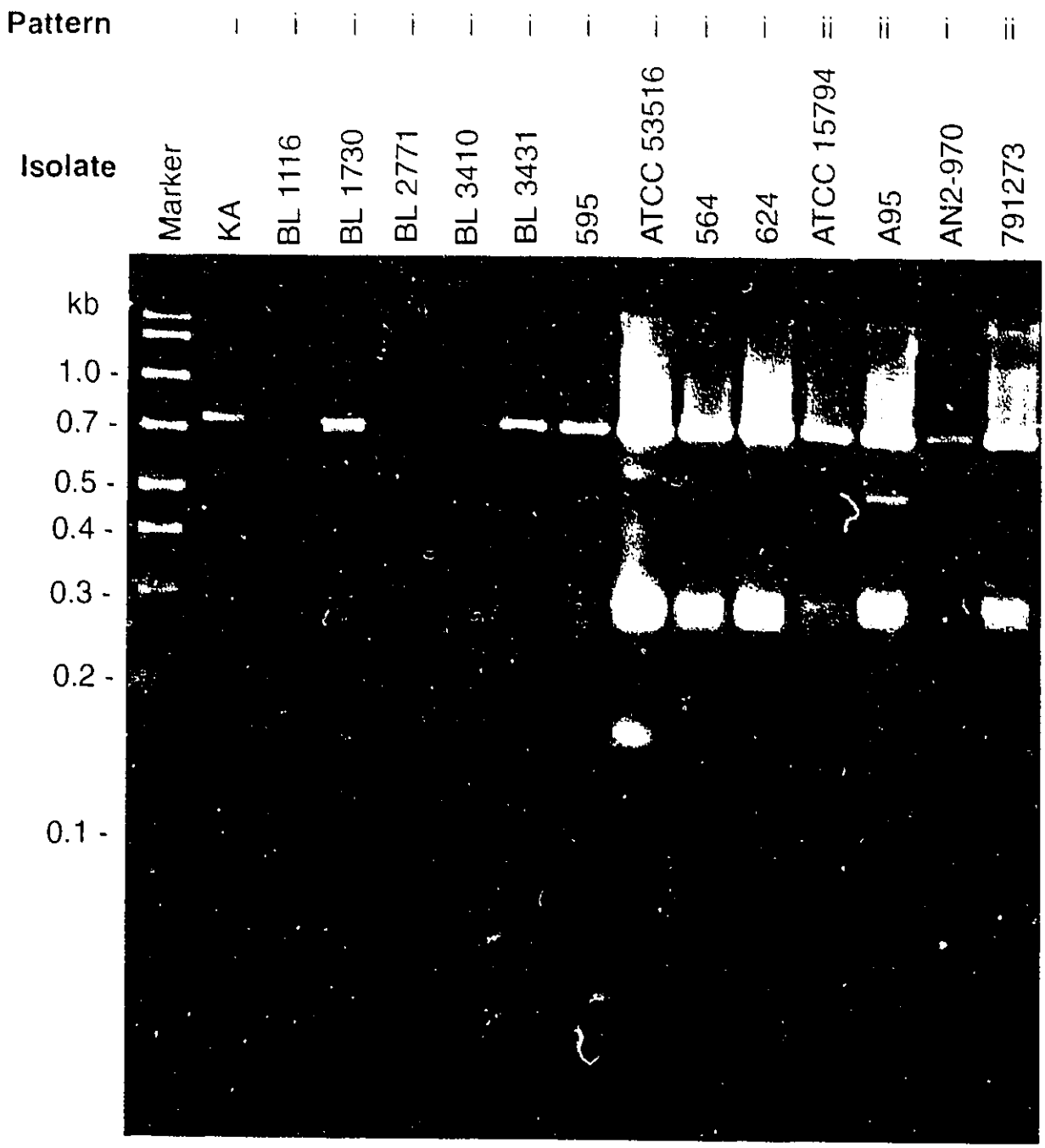

Pattern
Isolate
Marker
KA
BL 1116
BL 1730
BL 2771
BL 3410
BL 3431
595
ATCC 53516
564
624
ATCC 15794
A95
AN2-970
791273
kb
1.0 -
0.7 -
0.5 -
0.4 -
0.3 -
0.2 -
0.1 -

**Figure 7.** Ribotype analysis of *Hin*dIII digested genomic DNA of 14 *P. magnus* isolates using the *rrn*B operon of *E. coli* as the probe. There are 9 ribotype grouping patterns (a-i). Pattern a: KA, BL 1116, and BL 3431 ($L^-$ $A^+$). Pattern b: BL 1730, BL 2771, and BL 3410 ($L^-$ $A^+$). Pattern c: 595 ($L^-$ $A^-$). Pattern d: ATCC 53516 ($L^+$ $A^-$). Pattern e: 564 and 624 ($L^+$ $A^-$). Pattern f: ATCC 15794 ($L^-$ $A^+$). Pattern g: A95 ($L^-$ $A^+$). Pattern h: AN2-970 ($L^-$ $A^-$). Pattern i: 791273 ($L^-$ $A^-$). The DNA fragments at 4.4 kb and 4.5 kb for isolate 624 are partials.

| Pattern | a | a | b | b | b | a | c | d | e | e | f | g | h | i | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Isolate** | **KA** | **BL 1116** | **BL 1730** | **BL 2771** | **BL 3410** | **BL 3431** | **595** | **ATCC 53516** | **564** | **624** | **ATCC 15794** | **A95** | **AN2-970** | **791273** | kb Marker |
| | | | | | | | | | | | | | | | - 10.2 |
| | | | | | | | | | | | | | | | - 8.1 |
| | | | | | | | | | | | | | | | - 6.1 |
| | | | | | | | | | | | | | | | - 5.1 |
| | | | | | | | | | | | | | | | - 4.1 |
| | | | | | | | | | | | | | | | - 3.1 |
| | | | | | | | | | | | | | | | - 2.0 |
| | | | | | | | | | | | | | | | - 1.6 |

**Table 11.** Ribotype banding patterns of genomic DNA digested with *Hin*dIII from 14 *P. magnus* isolates.

| Fragment size | Banding pattern fragment | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| (kb)[a, b] | a | b | c | d | e | f | g | h | i |
| 9.8 ± 0.61 | | | | | | | | + | |
| 9.3 ± 1.21 | | | + | | | | | | |
| 8.6 ± 0.20 | | | | | | | + | | + |
| 8.0 ± 0.65 | + | + | | | | + | | | |
| 7.9 ± 0.85 | | | | | | | | + | |
| 7.5 ± 0.51 | | + | | | | | | | + |
| 7.0 ± 0.56 | | | | + | + | + | + | | |
| 6.4 ± 0.63 | + | | | + | + | | | | |
| 5.9 ± 0.45 | | | + | | | | | | |
| 5.8 ± 0.22 | | | | | | | | | + |
| 4.7 ± 0.35 | + | + | | | | | | | |
| 4.6 ± 0.10 | | | | | | | | + | + |
| 4.5 ± 0.10 | | | | | | | | + | |
| 4.4 ± 0.26 | | + | | | | | | | |
| 4.2 ± 0.33 | + | | | | | | | | |
| 4.0 ± 0.09 | | | + | | | | + | | |
| 3.8 ± 0.25 | | | | + | + | | | | |
| 3.6 ± 0.12 | + | | + | | | | + | + | + |
| 3.5 ± 0.24 | | + | | | | | | | |
| 3.4 ± 0.13 | | | | | | | + | + | + |
| 3.2 ± 0.14 | | + | + | | | + | | | |
| 3.1 ± 0.12 | + | | | | | | | | |
| 3.0 ± 0.10 | | | | | | | | + | |
| 2.8 ± 0.12 | | | + | + | | + | + | | |
| 2.65 ± 0.07 | | | | | | | | + | |
| 2.6 ± 0.08 | | | + | + | + | + | + | | + |
| 2.5 ± 0.07 | + | + | | + | + | + | | + | |
| 2.4 ± 0.08 | | | + | | + | | | | |
| 2.3 ± 0.0 | | | | | | + | + | + | |
| 1.9 ± 0.10 | | | | | + | | | | |
| 1.4 ± 0.10 | | | | | + | | | | |

[a] Average values of 3 replicates for each of the 14 *P. magnus* isolates except for a and b (3 replicates of 3 isolates), e (3 replicates of 2 isolates) and g, h, and i (2 replicates of 1 isolate).

[b] See Table 10.

a- KA, BL 1116, BL 3431, b- BL 1730, BL 2771, BL 3410, c- 595, d- ATCC 53516, e- 564 and 624, f- ATCC 15794; g- A95; h- AN2-970; i- 791273.

distinctive ribotype patterns containing seven to ten bands (Figure 7, pattern c, h-i).

Ribotyping of *Eco*RI digests of genomic DNA from the same 14 *P. magnus* isolates also produced the same nine ribotype groups (a to i) (Table 12 and 14); sizes of the *Eco*RI fragments ranged from 1.3-11.3 kb (data not shown). The fragment sizes obtained for *Eco*RI-based ribotypes of the fourteen *P. magnus* isolates are listed (Table 12). The three $L^+$ $A^-$ isolates were typed into two patterns, d and e (Table 12) which contained eleven common bands: strain ATCC 53516 comprised pattern d while isolates 564 and 624 belonged to pattern e. The eight $L^-$ $A^+$ isolates resolved into 4 patterns (Table 12) with Swedish isolates KA, BL 1116, and BL 3431 belonging to pattern a, Swedish isolates BL 1730, BL 2771, and BL 3410 belonging to pattern b and type strain ATCC 15794 and Canadian isolate A95 having distinctive patterns f and g. Pattern a contained ten bands and pattern b contained thirteen bands with both patterns sharing five common bands. Species type strain ATCC 15794 had nine bands (pattern f) and shared three common bands with isolate A95 and four with BL 1730, BL 2771, and BL 3410 (pattern b). Canadian isolate A95 had nine bands (pattern g) and shared four common bands with BL 1730, BL 2771, and BL 3410 (pattern b). The three $L^-$ $A^-$ isolates 595, AN2-970, and 791273 each had distinctive ribotype patterns containing nine to ten bands (pattern c, h-i). All ribotyping patterns were reproducibly replicated three times with each isolate.

**3.8 PULSED-FIELD GEL ELECTROPHORESIS.**

PFGE has proven to be a discriminatory typing method and may be the most discriminatory genotyping method available depending on the species analyzed (8). PFGE was chosen to show the genetic make-up of *P. magnus* $L^+$ and $A^+$ isolates.

*Xho*I-digestion subdivided the 14 *P. magnus* isolates into ten patterns accordingly (277)

**Table 12.** Ribotype banding patterns of genomic DNA digested with *Eco*RI from 14 *P. magnus* isolates.

| | Banding fragment pattern | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Fragment Size[a, b] (kb) | a | b | c | d | e | f | g | h | i |
| 11.3 ± 0.46 | | + | | | | | | | |
| 9.3 ± 0.70 | | | | | | + | + | | |
| 8.6 ± 1.04 | | | | | + | | | | |
| 8.5 ± 0.75 | + | + | | | | | | | |
| 7.1 ± 0.74 | | | | + | | | | | + |
| 6.4 ±0.68 | + | | + | | | | | | |
| 6.3 ± 0.38 | | + | | | | | | | |
| 5.6 ± 0.37 | | | | + | + | | | | |
| 5.5 ± 0.25 | | + | | | | | | | |
| 5.0 ± 0.47 | | | | + | + | + | + | + | |
| 4.9 ± 0.36 | + | | | | | | | | |
| 4.8 ± 0.38 | | + | | | | + | + | | |
| 4.6 ± 0.50 | | | | | | | | | + |
| 4.5 ± 0.28 | | + | | + | + | | | + | |
| 4.4 ± 0.13 | + | | | | | + | + | | |
| 4.3 ± 0.26 | + | + | | | | | | | |
| 4.1 ± 0.30 | | | + | | | | | | |
| 4.0 ± 0.48 | + | | | | + | | | + | |
| 3.9 ± 0.42 | | + | | | | | | | |
| 3.8 ± 0.26 | | | | | | + | | + | |
| 3.6 ± 0.29 | | | + | | | | | | + |
| 3.5 ± 0.24 | + | + | + | + | + | | | | |

Table 12 continued.

| | Banding pattern fragment | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Fragment Size[a, b] (kb) | a | b | c | d | e | f | g | h | i |
| 3.2 ± 0.07 | | | + | | | | | | |
| 3.0 ± 0.07 | | | | | | | + | | |
| 2.8 ± 0.18 | | | + | + | + | | | | |
| 2.7 ± 0.13 | + | + | + | + | + | + | + | + | + |
| 2.6 ± 0.17 | | | | + | + | | | | |
| 2.5 ± 0.13 | + | + | + | | | | + | + | + |
| 2.4 ± 0.18 | | | | + | + | + | | + | |
| 2.2 ± 0.17 | | | | | | + | | | |
| 2.0 ± 0.12 | | + | | | | | | | |
| 1.8 ± 0.21 | | | | | | | | | + |
| 1.6 ± 0.07 | | | + | | | | | | |
| 1.4 ± 0.07 | | | | | | | + | | + |
| 1.3 ± 0.07 | + | + | + | + | + | + | + | + | + |

[a] Average values of 3 replicates for each of the 14 *P. magnus* isolates except for a and b (3 replicates of 3 isolates), and e (3 replicates of 2 isolates).
a- KA, BL 1116, BL 3431, b- BL 1730, BL 2771, BL 3410, c- 595, d- ATCC 53516, e- 564, and 624, f- ATCC 15794; g- A95; h- AN2-970; i- 791273.

[b] See Table 10.

(Figure 8 and Table 13). The three $L^-$ $A^-$ isolates (ATCC 53516, 564, and 624) had three unique patterns, D-F (Figure 8). The eight $L^-$ $A^+$ isolates were differentiated into four patterns: KA, BL 1116, and BL 3431, were typed as profile A (Figure 8); isolates BL 1730, BL 2771, and BL 3410 were typed as profile B (Figure 8); and isolates ATCC 15794 and A95 were typed as profiles G and H, respectively (Figure 8). Also, the $L^-$ $A^-$ isolates 595, AN2-970 and 791273 each had unique profiles designated C, I and J, respectively (Figure 8). PFGE grouping patterns yielded more bands (13 to 20) per isolate as compared to the *Hin*dIII- or *Eco*RI- based ribotypes (6 to 11 bands). The fragment sizes obtained after PFGE of *Xho*I digestion of agarose slices from the fourteen *P. magnus* isolates are listed (Table 13).

The banding patterns for the three genotyping methods for all the strains are summarized in Table 14. Combining all three methods, the protein L-containing isolates ($L^+$ $A^-$) could be divided into three banding pattern groups (V to VII); the $L^-$ $A^+$ isolates could be divided into four banding pattern groups (I to IV); and the $L^-$ $A^-$ isolates could be divided into three banding pattern groups (VIII to X) (Table 14).

All three genotyping techniques were 100% reproducible and 100% typeable for the 14 *P. magnus* isolates. PCR-REA of the 16S rRNA gene was not discriminating after digestion with one of *Alu*I, *Hin*fI, *Hpa*II, and *Sty*I for the 14 *P. magnus* isolates (1 banding pattern) whereas after digestion with *Sau*3A1 ($D = 0.3626$) and *Taq*I ($D = 0.4945$), there was low discrimination resulting in two banding patterns (Table 15). Ribotyping after digestion of chromosomal DNA from the 14 *P. magnus* isolates with *Hin*dIII and *Eco*RI resulted in nine banding patterns each. The index of discrimination was 0.9231 (Table 15). PFGE was the most discriminating method resulting in ten banding patterns for the 14 *P. magnus* isolates. The index of discrimination was 0.9341 (Table 15).

**Figure 8.** PFGE of *Xho*I digested genomic DNAs from 14 *P. magnus* isolates. There are 10 banding group patterns (A-J). Pattern A: KA, BL 1116, and BL 3431 ($L^-$ $A^+$). Pattern B: BL 1730, BL 2771, and BL 3410 ($L^-$ $A^+$). Pattern C: 595 ($L^-$ $A^-$). Pattern D: ATCC 53516 ($L^+$ $A^-$). Pattern E: 564 ($L^+$ $A^-$). Pattern F: 624 ($L^+$ $A^-$). Pattern G: ATCC 15794 ($L^-$ $A^+$). Pattern H: A95 ($L^-$ $A^+$). Pattern I: AN2-970 ($L^-$ $A^-$). Pattern J: 791273 ($L^-$ $A^-$). Marker: lambda ladder (48.5-1,018 kb; Promega). The 81.3 kb band seen with KA and BL 1116 were resolved into 2 bands, similar to BL 3431(→) under modified conditions.

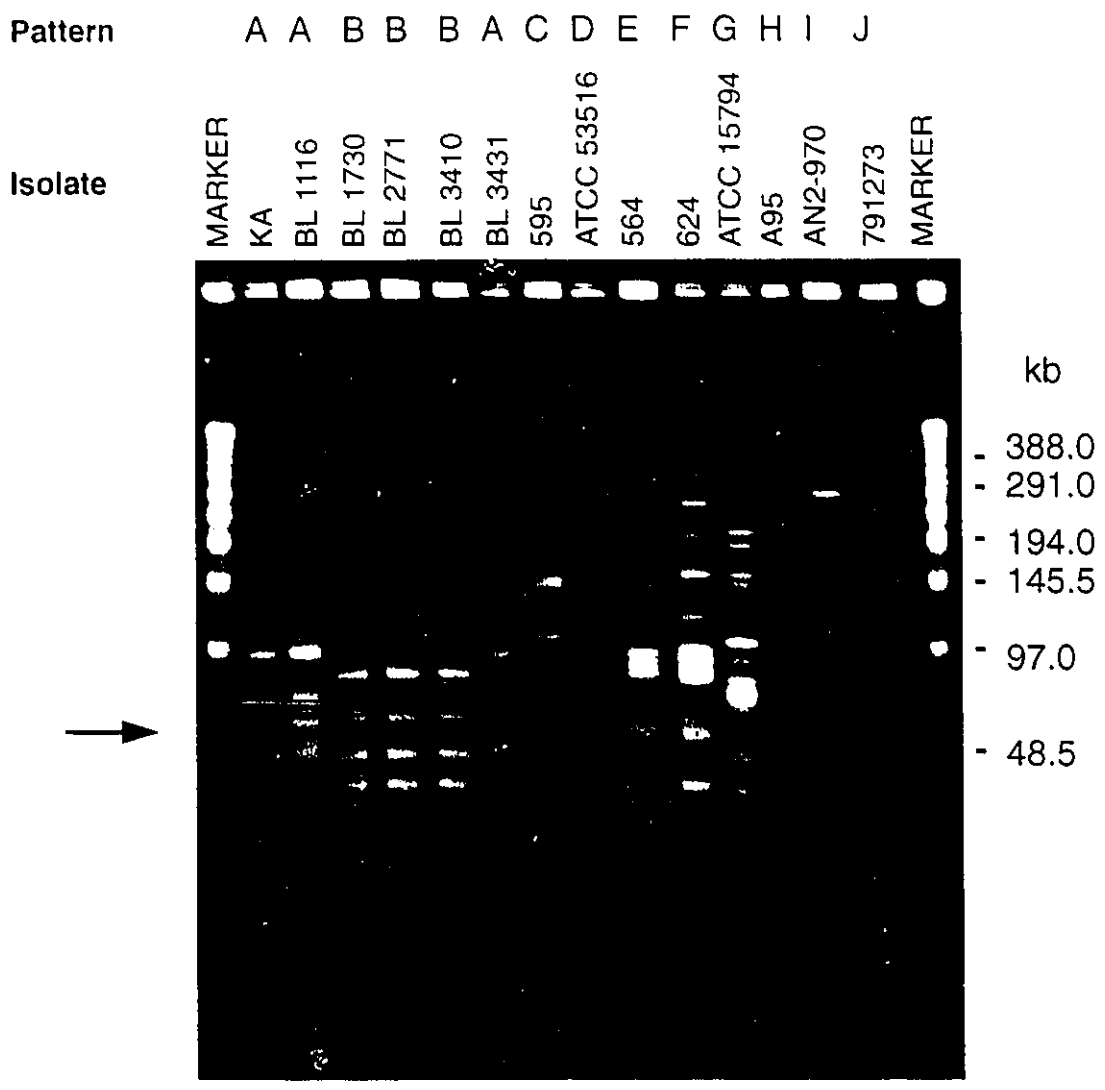

Pattern
A A B B B A C D E F G H I J
Isolate
MARKER
KA
BL 1116
BL 1730
BL 2771
BL 3410
BL 3431
595
ATCC 53516
564
624
ATCC 15794
A95
AN2-970
791273
MARKER
kb
388.0
291.0
194.0
145.5
97.0
48.5

**Table 13.** Pulsed-field gel electrophoresis of *Xho*I digested genomic DNA from 14 *P. magnus* isolates.

| | Banding fragment pattern | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Fragment Size[a] (kb) | A | B | C | D | E | F | G | H | I | J |
| 421.2 | | | + | | | | | | | |
| 271.3 | + | | | | | | | | | |
| 262.3 | | | | | | | | | + | |
| 253.8 | | | | + | + | + | | | | |
| 238.1 | | | + | | | | | | + | |
| 224.0 | | | | | | | | + | | |
| 217.5 | + | + | | | | + | | | | |
| 211.3 | | | | | | | | + | | |
| 205.3 | | | | | | | + | | | |
| 199.6 | | | | | + | + | | | | |
| 189.0 | | + | | + | | | + | | | |
| 179.3 | + | | | + | + | + | | | | |
| 170.3 | | | | | + | | | + | | + |
| 158.1 | | | | | | | + | | | |
| 154.4 | | | | | | | | + | | + |
| 150.8 | | | | + | + | + | | | | |
| 147.2 | | | + | | | | | | | + |
| 143.9 | | | + | | | | + | | | |
| 140.6 | | | | | | | | + | | |
| 134.4 | | | | + | + | + | | | | + |
| 131.5 | | + | | | | | | | | |
| 128.6 | | | | | | | | | | + |

**Table 13 continued.**

| Fragment Size[a] (kb) | Banding fragment pattern | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J |
| 123.2 | | | | | | | | + | | |
| 118.1 | | | | | | + | | | + | |
| 115.6 | | | | + | | | | | | |
| 113.3 | + | + | | | | | | | + | |
| 110.0 | | | | | + | | | | | |
| 108.7 | | | + | | | | + | | + | |
| 104.4 | | + | | | | | | | | |
| 100.4 | + | | | | + | + | | | | + |
| 98.4 | | + | | | | | + | | | |
| 96.6 | | | + | + | + | + | | | | |
| 92.9 | + | | | + | + | + | | + | | |
| 91.2 | | + | | | | | | | | |
| 89.4 | | | | | | | + | + | | + |
| 87.8 | | | | + | | | | | | |
| 84.6 | | + | + | | | | + | | | |
| 83.0 | + | | | | | | | | | |
| 81.5 | | | | | | | + | | | |
| 80.0 | | | + | | | | | | | + |
| 78.6 | | | | | | | + | | + | |
| 75.8 | + | | | | | | | | | |
| 74.4 | + | + | + | | | | | | + | + |
| 73.1 | | | | | | | + | | | + |

**Table 13 continued.**

| Fragment Size[a] (kb) | Banding fragment pattern | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J |
| 71.8 | | | | | + | + | | | | |
| 70.5 | | | + | | | | | | | |
| 69.3 | + | | | + | + | + | | | + | |
| 66.9 | | | + | | | | | + | | |
| 65.8 | | | | + | + | | | | | |
| 64.6 | + | + | | | | | + | + | | |
| 63.5 | | | | | | | | | | + |
| 62.4 | + | | | | | | | | | |
| 61.3 | | + | | | | | | | | |
| 58.2 | | | + | | | | | | + | |
| 57.2 | + | + | | | | | | + | | |
| 56.3 | | | + | + | + | + | | | | |
| 53.5 | | | | | | | + | | | |
| 52.6 | + | | | | | | | | | + |
| 50.8 | | + | | | | | | + | | + |
| 49.1 | | | | | | | | | + | |
| 47.5 | | | | | | | | | + | |
| 46.7 | | + | | | | | | | | + |
| 45.9 | | | | + | + | + | | | | |
| 45.1 | + | | + | | | | | | | |
| 44.4 | | | | | | | + | | + | |
| 43.6 | | | | | | | + | + | | |

**Table 13 continued.**

| Fragment Size[a] (kb) | Banding fragment pattern | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J |
| 42.1 | + | | | | | | + | | | |
| 41.4 | | | | | | | | + | | |
| 40.0 | | + | | | | | | + | + | + |
| 39.4 | + | | | | | | | | | |
| 37.4 | + | | | | | | + | | | |
| 36.1 | | | | | | | + | | | |
| 32.6 | | | | + | + | + | + | | | |
| 30.4 | | | | | | | + | | | |

[a] One replicate for each of the 14 *P. magnus* isolates except for a and b (1 replicate of 3 isolates).
A- KA, BL 1116, BL 3431, B- BL 1730, BL 2771, BL 3410, C- 595, D- ATCC 53516, E- 564; F- 624, G- ATCC 15794; H- A95; I- AN2-970; J- 791273.

**Table 14.** The banding patterns of 14 *P. magnus* isolates using 3 different typing methods resulted in 10 banding pattern groups.

| Phenotype[a] | Strain No. | Banding pattern of PCR-REA[b] *Taq*I | *Sau*3A1 | Ribotyping *Hin*dIII or *Eco*RI | PFGE *Xho*I | Banding group pattern |
|---|---|---|---|---|---|---|
| $L^-A^+$ | KA, BL 1116, BL 3431 | 1 | i | a | A | I |
| | BL 1730, BL 2771, BL 3410 | 1 | i | b | B | II |
| | ATCC 15794 | 1 | ii | f | G | III |
| | A95 | 1 | ii | g | H | IV |
| $L^+A^-$ | ATCC 53516 | 2 | i | d | D | V |
| | 564 | 2 | i | e | E | VI |
| | 624 | 2 | i | e | F | VII |
| $L^-A^-$ | 595 | 2 | i | c | C | VIII |
| | AN2-970 | 2 | i | h | I | IX |
| | 791273 | 1 | ii | i | J | X |

[a] Protein L-containing ($L^+$); albumin-binding ($A^+$).

[b] Polymerase chain reaction (PCR)-restriction endonuclease analysis (REA) of the 16S rRNA genes.

**Table 15.** Simpson's index of discrimination for 3 genotyping methods (PCR-REA, ribotyping, and pulsed-field gel elcctrophoresis).

| Method | Restriction endonuclease | No. of types | Size (%) of largest type | Simpson's index of discrimination (*D*) |
|---|---|---|---|---|
| PCR-REA | *Sau*3A1 | 2 | 78.6 | .3626 |
| | *Taq*I | 2 | 64.3 | .4945 |
| Ribotyping | *Eco*RI/*Hin*dIII | 9 | 21.4 | .9231 |
| Pulsed-field gel electrophoresis | *Xho*I | 10 | 21.4 | .9341 |

### 3.9 STATISTICAL ANALYSIS.

Based on the combined *F* values (Table 16), cluster analysis was performed with ribotype results including *Eco*RI and *Hin*dIII digests and *Xho*I digests of PFGE products to obtain a dendrogram (Figure 9) to show the relationship of the 14 isolates. The *P. magnus* $L^+A^-$ isolates 564 and 624 (94.3% similarity) were more closely related to each other than to $L^+A^-$ strain ATCC 53516 (73.9% similarity). These 3 $L^+A^-$ Swedish isolates were more related among themselves than to other isolates forming Cluster A (Figure 9). The $A^+$ phenotype was found in a more diverse genetic background than the $L^+$ phenotype. The 6 $L^-A^+$ isolates from Sweden fell into two Clusters; Cluster B (KA, BL 1116 and BL 3431; Figure 9) and Cluster C (BL 1730, BL 2771 and BL 3410; Figure 9). Isolates in Cluster B and Cluster C had only 40% similarity between them and less with others including the three $L^+$ $A^-$ isolates as well as the remaining $L^-$ $A^+$ isolates, A95 and the type strain ATCC 15794. The latter 2 isolates were also unrelated to each other. All 3 clusters were isolates obtained from Swedish patients. Also, there was little similarity amongst the $L^-$ $A^-$ isolates.

The dendrograms based on cluster analysis of the *F* values (Table 17) from the *Hin*dIII-based ribotypes of the 14 *P. magnus* isolates (Figure 10) and the *F* values (Table 18) from the *Eco*RI-based ribotypes of the 14 *P. magnus* isolates (Figure 11) are shown. The dendrogram based on the *F* values (Table 19) of PFGE of the *Xho*I DNA digests for the 14 *P. magnus* isolates is shown in Figure 12. These three cluster analysis' showed some similar trends as observed with the combined data (Figure 9). The three sets of clusters comprised the same set of isolates with the remaining isolates forming separate taxonomic units. However, the relatedness of each taxonomic unit showed some variation which is reflected in their *F* values and the method.

**Table 16.** Combined *F* values from ribotyping and PFGE[a] experiments. Banding pattern group I-X[b].

| | I | II | III | IV | V | VI | VII | VIII | IX | X |
|---|---|---|---|---|---|---|---|---|---|---|
| I | | .4000 | .2253 | .2388 | .2462 | .2857 | .3143 | .2388 | .2985 | .2424 |
| II | | | .2817 | .2388 | .1846 | .1429 | .1714 | .2090 | .2985 | .2727 |
| III | | | | .3824 | .3030 | .2253 | .2253 | .2353 | .2941 | .1493 |
| IV | | | | | .2258 | .2090 | .1791 | .2813 | .2500 | .3809 |
| V | | | | | | .7385 | .7385 | .2580 | .2258 | .1639 |
| VI | | | | | | | .9429 | .2388 | .2388 | .1818 |
| VII | | | | | | | | .2388 | .2388 | .1515 |
| VIII | | | | | | | | | .2500 | .3492 |
| IX | | | | | | | | | | .2857 |
| X | | | | | | | | | | - |

[a] Ribotyping results include *Eco*RI and *Hin*dIII genomic digests. PFGE includes analysis of *Xho*I products.

[b] Banding group patterns: I- KA, BL 1116, BL 3431; II- BL 1730, BL 2771, BL 3410; III- ATCC 15794; IV- A95; V- ATCC 53516; VI- 564; VII- 624; VIII- 595; IX- AN2-970; X- 791273.

**Figure 9.** Dendrogram of 14 *P. magnus* isolates using the *F* values calculated from *Hind*III and *Eco*RI generated ribotypes and PFGE patterns.

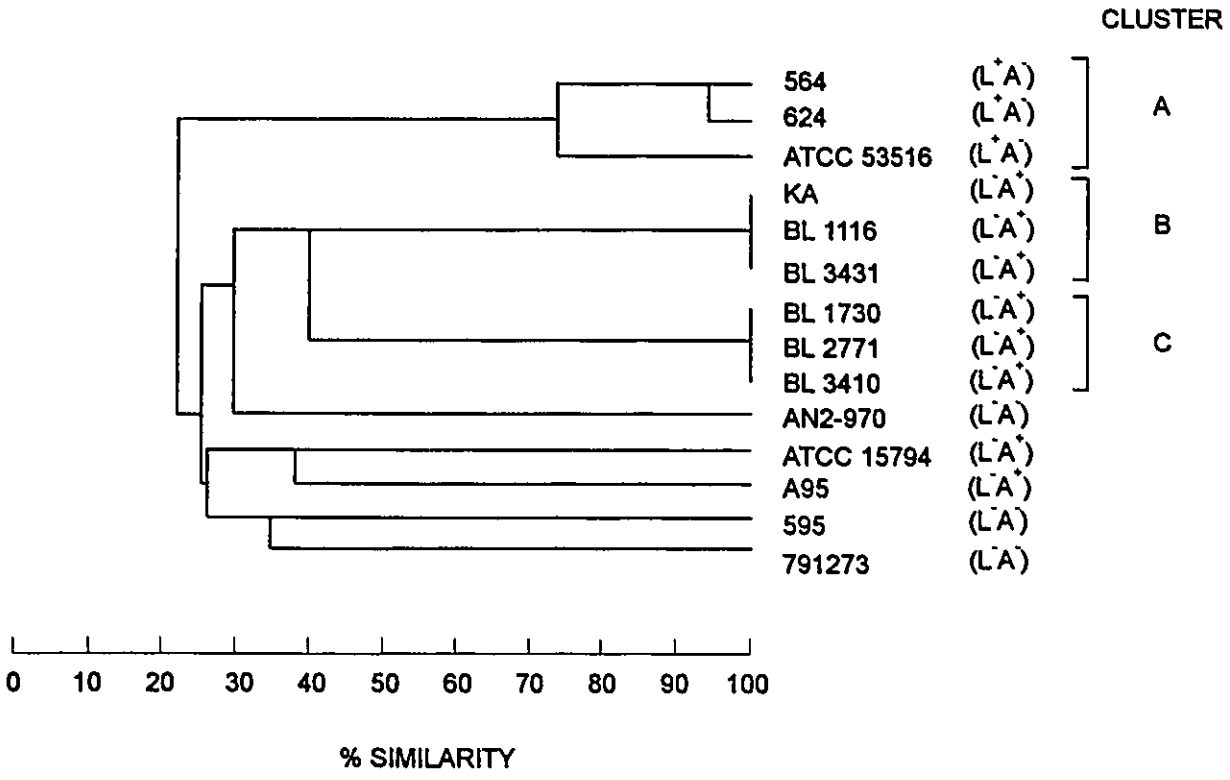

CLUSTER
564 (L+A-)
624 (L+A-)
ATCC 53516 (L+A-)
KA (L-A+)
BL 1116 (L-A+)
BL 3431 (L-A+)
BL 1730 (L-A+)
BL 2771 (L-A+)
BL 3410 (L-A+)
AN2-970 (L-A-)
ATCC 15794 (L-A+)
A95 (L-A+)
595 (L-A-)
791273 (L-A-)
A
B
C
0 10 20 30 40 50 60 70 80 90 100
% SIMILARITY

**Table 17.** *F* values of *Hin*dIII-based ribotypes for 14 *P. magnus* isolates. Banding pattern group I-X[a].

| | I | II | III | IV | V | VI | VII | VIII | IX | X |
|---|---|---|---|---|---|---|---|---|---|---|
| I | | .4286 | .2857 | .1333 | .3077 | .2667 | .2667 | .1333 | .3529 | .2857 |
| II | | | .4286 | .0000 | .1538 | .1333 | .1333 | .1333 | .2529 | .2857 |
| III | | | | .5333 | .6154 | .4000 | .4000 | .4000 | .2353 | .1429 |
| IV | | | | | .4286 | .2500 | .2500 | .6250 | .3333 | .5333 |
| V | | | | | | .7143 | .7143 | .2857 | .1250 | .1538 |
| VI | | | | | | | 1.000 | .2500 | .1111 | .1333 |
| VII | | | | | | | | .2500 | .1111 | .1333 |
| VIII | | | | | | | | | .1111 | .5333 |
| IX | | | | | | | | | | .3529 |
| X | | | | | | | | | | - |

[a]I- KA, BL 1116, BL 3431; II- BL 1730, BL 2771, BL 3410; III- ATCC 15794; IV- A95; V- ATCC 53516; VI- 564; VII- 624; VIII- 595; IX- AN2-970; X- 791273.

**Figure 10.** Dendrogram of 14 *P. magnus* isolates using the calculated *F* values after *Hin*dIII diigestion of genomic DNA and probing with the *rrn*B operon of *E. coli*.

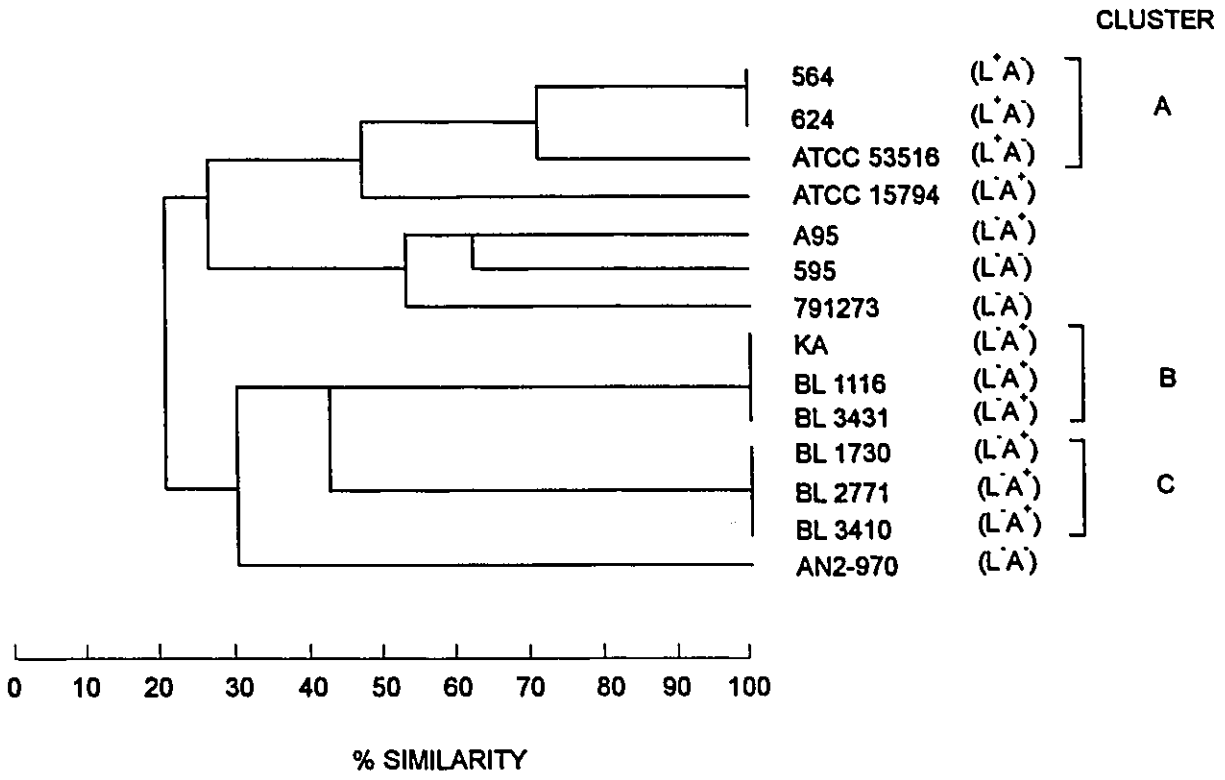

CLUSTER
564 (L+A-)
624 (L+A-)
ATCC 53516 (L+A-)
ATCC 15794 (L-A+)
A95 (L-A+)
595 (L-A-)
791273 (L-A-)
KA (L-A+)
BL 1116 (L-A+)
BL 3431 (L-A+)
BL 1730 (L-A+)
BL 2771 (L-A+)
BL 3410 (L-A+)
AN2-970 (L-A-)
A
B
C
0 10 20 30 40 50 60 70 80 90 100
% SIMILARITY

**Table 18.** *F* values of *Eco*RI-based ribotypes for 14 *P. magnus* isolates. Banding pattern group I-X[a].

| | I | II | III | IV | V | VI | VII | VIII | IX | X |
|---|---|---|---|---|---|---|---|---|---|---|
| I | | .5217 | .3158 | .4210 | .3000 | .3809 | .3809 | .5000 | .4210 | .3158 |
| II | | | .2727 | .3636 | .3478 | .3333 | .3333 | .3478 | .3636 | .2727 |
| III | | | | .6667 | .4210 | .4000 | .4000 | .2105 | .5556 | .2222 |
| IV | | | | | .3158 | .3000 | .3000 | .3158 | .4444 | .3333 |
| V | | | | | | .7619 | .7619 | .4000 | .5263 | .3158 |
| VI | | | | | | | 1.000 | .3809 | .6000 | .2000 |
| VII | | | | | | | | .3809 | .6000 | .2000 |
| VIII | | | | | | | | | .3158 | .4210 |
| IX | | | | | | | | | | .4444 |
| X | | | | | | | | | | - |

[a] I- KA, BL 1116, BL 3431; II- BL 1730, BL 2771, BL 3410; III- ATCC 15794; IV- A95; V- ATCC 53516; VI- 564; VII- 624; VIII- 595; IX- AN2-970; X- 791273.

**Figure 11.** Dendrogram of 14 *P. magnus* isolates using the calculated *F* values after *Eco*RI digestion of genomic DNA and probing with the *rrn*B operon of *E. coli*.

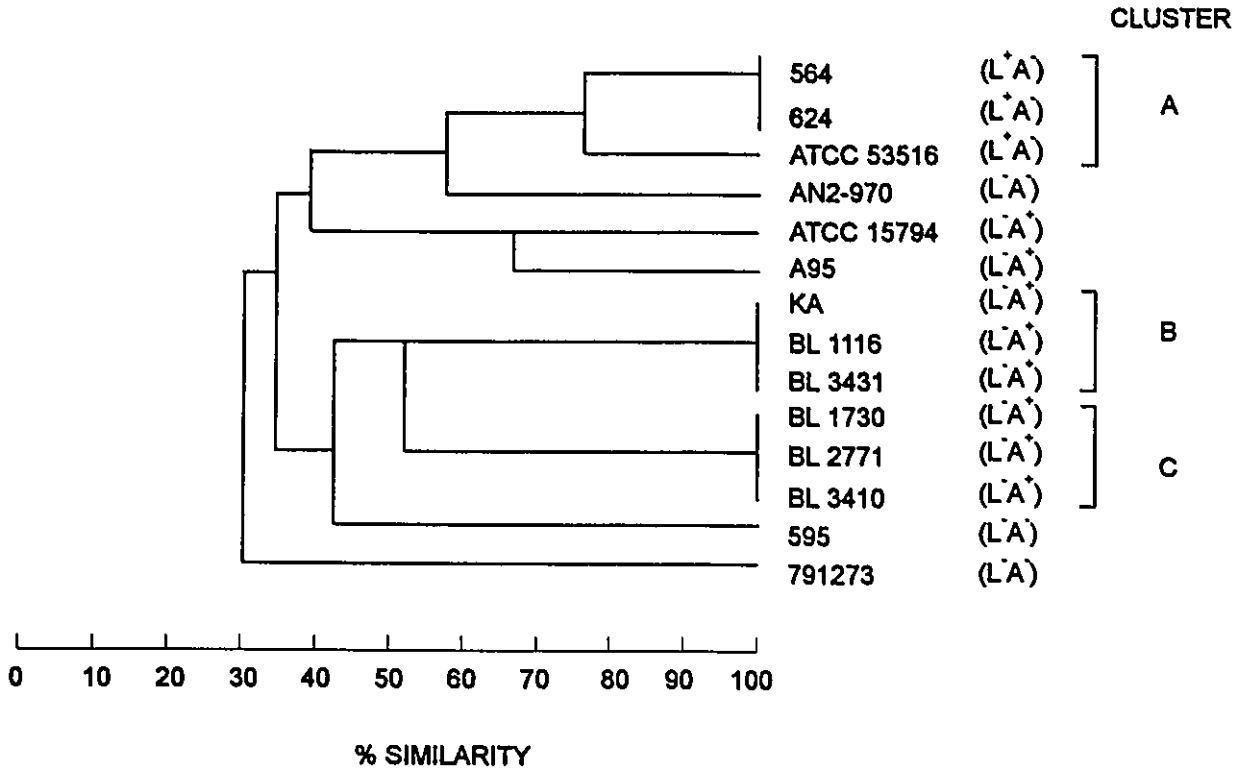
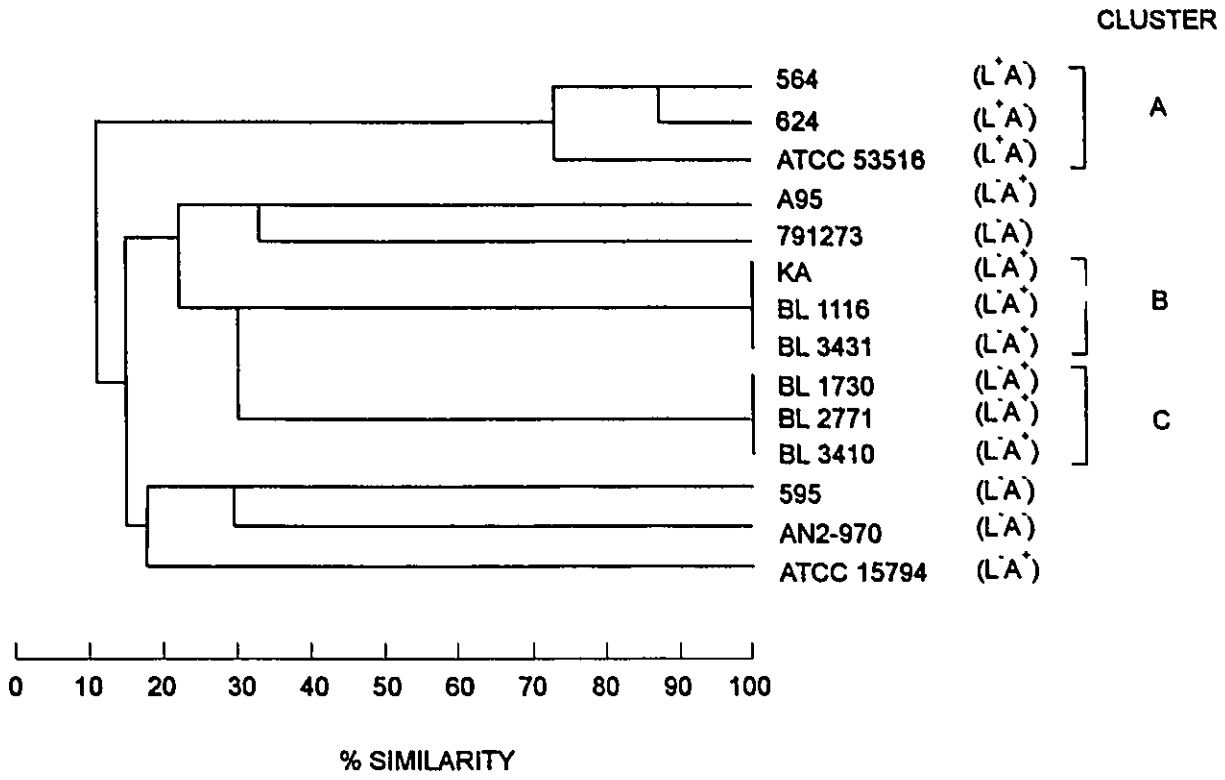

CLUSTER
564 (L+A-)
624 (L+A-)
ATCC 53516 (L+A-)
AN2-970 (L-A-)
ATCC 15794 (L-A+)
A95 (L-A+)
KA (L-A+)
BL 1116 (L-A+)
BL 3431 (L-A+)
BL 1730 (L-A+)
BL 2771 (L-A+)
BL 3410 (L-A+)
595 (L-A-)
791273 (L-A-)
A
B
C
0 10 20 30 40 50 60 70 80 90 100
% SIMILARITY

**Table 19.** *F* values of *Xho*I digested DNA of 14 *P. magnus* isolates after pulsed-field gel electrophoresis. Banding pattern group I-X[a].

| | I | II | III | IV | V | VI | VII | VIII | IX | X |
|---|---|---|---|---|---|---|---|---|---|---|
| I | | .3030 | .1579 | .1818 | .1875 | .2353 | .2941 | .1250 | .1935 | .1818 |
| II | | | .2286 | .2667 | .0690 | .0000 | .0645 | .1379 | .2143 | .2667 |
| III | | | | .1714 | .1176 | .0556 | .0556 | .1765 | .1818 | .1143 |
| IV | | | | | .0690 | .1290 | .0645 | .0690 | .0714 | .3333 |
| V | | | | | | .7333 | .7333 | .1429 | .0741 | .0690 |
| VI | | | | | | | .8750 | .1333 | .0690 | .1935 |
| VII | | | | | | | | .1333 | .0690 | .1290 |
| VIII | | | | | | | | | .2963 | .2069 |
| IX | | | | | | | | | | .1429 |
| X | | | | | | | | | | - |

[a]I- KA, BL 1116, BL 3431; II- BL 1730, BL 2771, BL 3410; III- ATCC 15794; IV- A95; V- ATCC 535516; VI- 564; VII- 624; VIII- 595; IX- AN2-970; X- 791273.

**Figure 12.** Dendrogram of 14 *P. magnus* isolates using the calculated *F* values after PFGE of *Xho*I digestion of DNA plugs.


CLUSTER
564
624
ATCC 53516
A95
791273
KA
BL 1116
BL 3431
BL 1730
BL 2771
BL 3410
595
AN2-970
ATCC 15794
(L+A)
(L+A)
(L+A)
(L-A+)
(L-A)
(L-A+)
(L-A+)
(L-A+)
(L-A+)
(L-A+)
(L-A)
(L-A)
(L-A+)
A
B
C
0
10
20
30
40
50
60
70
80
90
100
% SIMILARITY

# 4. DISCUSSION

## 4.1 THE ARRAY OF AMINOPEPTIDASE ACTIVITIES OF *PEPTOSTREPTOCOCCUS* SPECIES IS CORRELATED WITH PROTEOLYTIC ACTIVITY.

Nonspecific protease activity has been observed in 3 *Peptostreptococcus* species isolated from the female genital tract but the nature of such activities has not been determined (167). Semi-quantitative analysis of the 4 *P. magnus* clinical isolates from ovarian and skin abscesses in this study showed weak proteolytic activity for five of the 11 substrates (gelatin, fibrinogen, BSA, casein, and autoclaved collagen) as compared to the proteolytic organism *P. gingivalis* W83, which is known to have at least 4 proteinases characterized (29, 76, 131, 223). The weak proteolytic activities for the 4 *P. magnus* clinical isolates suggests that it is due to nonspecific proteases (e.g. gelatinase on autoclaved collagen). I propose that proteolytic activity observed for these *P. magnus* isolates may be due to nonspecific proteases such as aminopeptidases for the following reasons. The majority (86.4%) of the 44 gelatin positive isolates were *P. magnus* and *P. micros*. Such weak proteolytic activities can be correlated with the aminopeptidase profiles of *P. magnus* and *P. micros* isolates which have more positive aminopeptidase reactions than *P. anaerobius, P. asaccharolyticus*, and *P. prevotii*. This is consistent with other studies that have shown a cluster of *P. magnus* isolates from nonpuerperal breast abscesses and diabetic foot infections have marked proteolytic activities which they claimed to produce collagenase and gelatinase using autoclaved collagen only as the substrate (127-128). Since autoclaved collagen is denatured to gelatin with some conformational variation, the proteolytic activity of *P. magnus* isolates from nonpuerperal breast abscesses and diabetic foot abscesses may in fact be collagenolytic which is similar to our *P. magnus* isolates (127-128). In

another study, oral isolates of *P. micros* have a wide range of peptidase activities which are likely involved in terminal glycoprotein degradation (278).

The distribution of certain positive aminopeptidase reactions among individual species detected by the rapid ID32A kit may be involved in proteolytic activities. The pre-formed enzyme profile from the rapid ID32A kit for *P. anaerobius* is consistent with previous reports where all isolates produced a proline aminopeptidase reaction (69, 182-184). All *P. asaccharolyticus* isolates produced ARG and HIS (69, 182). The *P. magnus* isolates and *P. micros* isolates had a wide array of positive aminopeptidase reactions which included all isolates having activity for ARG and LEU and most for PYR (69, 182-184). All *P. prevotii* isolates produced ARG but their overall aminopeptidase profiles were heterogeneous (69, 182-184). This suggests that certain positive aminopeptidase reactions that are always or nearly always present in a species profile may be important in proteolytic activities. For example, since *P. magnus* and *P. micros* account for most of the gelatin hydrolyzing isolates, ARG, LG, LEU, PYR, TYR, ALA, GLY, HIS, and SER may be important in these activities. Future work should involve the quantitiative and enzymatic analysis using fluorogenic or chromogenic substrates to spectrophotometrically assay for their corresponding activities. As well, besides the number and types of positive aminopeptidase reactions, it is possible that other factors such as the quantity, and/or activity of positive aminopeptidase reactions may contribute to the observed gelatin hydrolysis by *P. anaerobius* and other species.

Increased levels of proline aminopeptidase in the vaginal fluids of bacterial vaginosis patients has been postulated to be a product of the endogenous bacterial flora which also includes *P. anaerobius* and has been used as a reliable marker enzyme for BV (248). Recently, it has been shown that *P. anaerobius* is significantly associated with BV (101). Aminopeptidases may reflect a part of

the pathogenic role of *Peptostreptococcus* isolates in female genital tract conditions. Concerted aminopeptidase activity of *Peptostreptococcus* spp. isolated from the female genital tract may be involved in terminal protein (tissue) degradation as seen in periodontal disease by *P. micros* and may increase their growth and of other microbes (278). This has been speculated in BV where *G. vaginalis* may have enhanced the growth of the endogenous anaerobic flora due to metabolic activities (45). Preliminary work on the construction of genomic libraries of *Peptostreptococcus* were performed to screen for proteolytic enzymes and is cited in Appendix A.

## 4.2 IDENTIFICATION OF *PEPTOSTREPTOCOCCUS* ISOLATES.

Pre-formed enzyme kits have been found to be a reliable and discriminatory method for identifying most *Peptostreptococcus* species and can be used in routine laboratories (180, 182). The use of the rapid ID32A kit extends the validation of pre-formed enzyme kits in identifying *Peptostreptococcus* species. Since the taxonomic classification of *Peptostreptococcus* species is currently being revised, pre-formed enzyme profiles for certain isolates that cannot be identified may be useful in aiding in classification by other techniques. Previous studies have evaluated prototype rapid ID32A kits for the identification of a variety of obligate anaerobes including *Peptostreptococcus* species and pigmented *Bacteroides* spp. (122, 182-184, 294). The rapid ID32A kit (bioMérieux Vitek, Inc. Hazelwood, MI.) tests for saccharolytic and proteolytic enzymes by using chromogenic substrates (182). Previously, there were two prototypes of the rapid ID32A kit used to identify clinical strains of *Peptostreptococcus* spp. (182-184). The prototype API system was the original form of the kit (184) while the ATB 32A system, which differed from the prototype API kit by excluding 4 biochemical reactions (acidification of glucose, acidification of fructose, acidification of trehalose, and the catalase reaction), was the first commercially available form (122, 182, 294). The

rapid ID32A kit differs from the ATB 32A system only by containing a code book but no colour chart for reading endpoints and allows the option for automatic reading using computer programs. One previous study showed there was complete agreement in the identification of 30 type and reference strains of Gram positive anaerobic coccus (GPAC) including 8 *Peptostreptococcus* species ($n$=23) with the prototype rapid ID32A kits and the conventional Virginia Polytechnic Institute (VPI) method and in another study there was > 99 % agreement for 40 reference and 256 clinical isolates of GPAC, including 9 species of *Peptostreptococcus* ($n$=272) (182, 184, 267). The ATB 32A kit was found to be useful in differentiating strains within species in addition to classification (182).

In this study, the rapid ID32A kit was used to identify 5 *Peptostreptococcus* species isolated predominantly from the female genital tract. Our results showed an 80% agreement with conventional VPI methods, which is lower than the 100% reported in other studies (182-184). This is due to the identification of eight of 11 *P. micros* isolates from the female genital tract as *P. magnus* and to the failure to classify eleven of 12 *P. prevotii* isolates. Eight of the 11 *P. micros* isolates from the female genital tract were identified as *P. magnus* because they had no PRO, PHE, and GG activity. The lack of activity for PRO, PHE, and GG has been observed for other *P. magnus* isolates (182). This might suggest that certain *P. micros* isolates from the female genital tract have atypical enzyme profiles compared to other body sites or that the VPI method may be unreliable. Except for the type strain of *P. prevotii*, the other 22 *P. micros* isolates and *P. prevotii* isolates were not included in the previous studies (69, 182-184). *P. prevotii* has been shown to be heterogeneous based on pre-formed enzyme profiles and *P. prevotii* taxonomy requires further clarification (69, 182-184). Recently, three new species, *P. vaginalis*, *P. lacrimalis*, and *P. lactolyticus* have been identified from five independent DNA similarity groups that had been tentatively identified as *P. prevotii* by biochemical

tests (142). Only *P. vaginalis* was isolated from the female genital tract (142). Li *et al.* used the pre-formed enzyme kits API ZYM and AN-IDENT for identification purposes of 12 clinical strains that had been previously identified as *P. prevotii* but were genetically different from the type strain *P. prevotii* ATCC 27337 (142). Li *et al.* (142) found that 12 isolates that were biochemically identified as *P. prevotii* failed to hybridize with the type strain *P. prevotii* ATCC 27337 nor any other *Peptostreptococcus* type strains. Ten of the 12 *P. prevotii* isolates fell into three new species (*P. vaginalis*, *P. lacrimalis*, and *P. lactolyticus*). This reinforces the results in this work that the species *P. prevotii* are heterogeneous. The pre-formed enzyme profile has an advantage over the conventional VPI method because it provides an 8-digit profile specific for isolates and discriminates well between isolates (182). In general, most individual tests were highly reproducible upon re-testing, as long as the inoculum density was similar. However, some *P. prevotii* isolates' proteolytic enzyme reaction results varied upon re-testing because many were very weak. For example, the type strain *P. prevotii* ATCC 9321 was variable for LEU compared to 7 clinical isolates which had strong reactions. This could be a reflection on the particular enzyme reaction, the growth conditions, or the organism itself which may not have been properly identified by the "gold" standard method. TYR, ALA, PHE, and LG tests were the hardest to interpret because most often these reactions were variable (very weak positive) and had to be repeated up to 6 times.

The pre-formed enzyme profile from the rapid ID32A kit for *P. anaerobius* is consistent with previous reports where production of proline aminopeptidase and αGLU were good differential characteristics (69, 182-184). Like its prototype kits, rapid ID32A was 100% successful in identifying *P. asaccharolyticus* isolates. As in other studies, all *P. asaccharolyticus* isolates were indole positive, mannose negative, urease negative, alkaline phosphatase negative, α-glucosidase negative, β-

glucurunidase negative, and did not reduce nitrate, typical characteristics of this species (105, 182-184).

The identification and differentiation of *P. magnus* from *P. micros* may be difficult because it has been based on relying on cell size, GLC, and negative carbohydrate utilization results (104-105, 182, 267). Previous studies of prototype kits showed PAL activity was variable or minimal for *P. magnus* isolates and did not discriminate well between the two species (182-184).The production of alkaline phosphatase has been used to distinguish *P. micros* (+) from *P. magnus* (-) (104) and in this study nineteen of 24 *P. magnus* isolates had PAL activity. Using this criteria for confirmation, the isolates with positive alkaline phosphatase reactions should be classified as *P. micros*. Therefore, the PAL test from the rapid ID32A kit is either hard to interpret, the test is not definitive for classification or the VPI method is unreliable. Thus, identification results of *P. magnus* and *P. micros* from female genital tract isolates using the rapid ID32A kit support the idea that these two species are difficult to differentiate. Electrophoretic profiles have been used to differentiate the two species but is unrealistic for routine identification purposes (275) whereas pyrolysis mass spectrophotometry has been an effective technique to separate *P. magnus* and *P. micros* (150).

The pre-formed enzyme profiles of *P. magnus* isolates agrees well with previous prototype pre-formed enzyme profiles. There was complete agreement in the proper identification of the type strain *P. micros* ATCC 33270, the 5 breast abscess isolates, and the 2 clinical isolates of unknown origin between the VPI method and the rapid ID32A pre-formed enzyme profiles. All produced a strong PAL reaction.

Recently, in a collaborative study with A. Willems and M. D. Collins, the sequencing of part or all of the 16S rRNA genes from eight of the 11 *P. micros* female genital tract isolates that were

identified as *P. magnus* by the rapid ID32A kit (Willems, personal communication) was performed. It was shown that upon sequencing the first five hundred to 600 nucleotides of the 16S rRNA gene, there was only zero to three base pair differences among the eight isolates (Willems, personal communication). Upon sequencing the entire 16S rRNA gene sequence from one of these 8 *P. micros* isolates (VH8952), there was more similarity with the type strain *P. magnus* ATCC 15794 (98.3%) than with *P. micros* Gifu 7701 (88%) (Willems, personal communication). This supports my rapid ID32A enzyme profile results that these eight *P. micros* isolates resemble the species *P. magnus.*

The pre-formed enzyme profiles for type strain *P. prevotii* ATCC 9321 and the clinical isolates confirm that this is a very heterogeneous group (69, 142, 182-184). By examining published common pre-formed enzyme profiles (oxidase, peptidase and sugar fermentation reaction tests) in the two biochemical kits (API ZYM and AN-IDENT) with rapid ID32A (142), only one of the clinical *P. prevotii* isolates (801478) used in this study could be identified as belonging to any of the five DNA similarity groups including the three new species *P. vaginalis*, *P. lacrimalis*, and *P. lactolyticus*. Isolate 801478 was tentatively identified as *P. lacrimalis* but this was based only on 14 common pre-formed enzyme reactions. This could be a reflection of the biochemical kits used (each kit may have different formulations resulting in different performances including individual tests), the difference in the inoculum density, the pre-formed enzyme tests may be different or a reflection on the wide heterogeneity of isolates tentatively identified as *P. prevotii*. The latter reason is confirmed by our results in that only the type strain *P. prevotii* ATCC 9321 could be identified as *P. prevotii* based on the 8-digit analytical profile. We agree that further taxonomic work needs to be done on strains phenotypically similar to *P. prevotii*.

Many of the clinical isolates of *P. prevotii* that could not be properly identified in this study

have been tentatively identified as falling into recently formed species groups and subspecies groups (180; Murdoch, personal communication). *P. assacharolyticus* isolates are heterogeneous and have been divided into three different groups based on colonial and cellular morphology and indole production (180). Using pyrolysis mass spectrophotometry, the type strains of *P. prevotii/P. tetradius* form a discrete group showing strong saccharolytic activity and urease production (180). Tentative identification by D. A. Murdoch using the pre-formed enzyme profiles of rapid ID32A of clinical *P. prevotii* isolates are as follows: isolates A129, 801478, and 811556 are *P. asaccharolyticus* (indole-negative), isolates AN2-253, PID676H, and PID990B are members of the *P. prevotii/P. tetradius* group, and the isolate of unknown clinical origin 83-125 has been identified as *P. vaginalis*.

Unlike the rapid ID32A kit, colour charts were provided for the original prototype API kit and the ATB 32A prototype. Colour charts would greatly help in variable reactions by making it easier to read endpoints. In future modifications and evaluation of the kit, *P. productus*, *P. tetradius*, and the newly speciated *P. vaginalis*, *P. lacrimalis*, and *P. lactolyticus* should be included in the database (142).

## 4.3 GENETIC RELATIONSHIP OF PROTEIN L-CONTAINING AND ALBUMIN-BINDING *P. MAGNUS* ISOLATES.

*P. magnus* is considered an opportunistic pathogen and there are no established data on the prevalence of strain variability or specific pathogenic strain types of this species for different anaerobic infections. Our molecular typing data showed that protein L and albumin-binding *P. magnus* strains are associated with clusters of genotypes. Although *P. magnus* is found as part of the normal flora of the female genital tract, only specific strains may be associated with female genital tract infections. Protein L has been shown to be associated with BV isolates (116). Association of

specific genotypes with disease has been observed in other species (277) and most bacterial pathogens are clonal in nature (8). Protein L and albumin-binding markers as well as genotyping methods used in this study will be useful tools for identifying specific strain types for different vaginal infections.

Very few studies have dealt with the genetic relationship of pathogenic isolates of *Peptostreptococcus* probably because of its low virulence and its unknown role in polymicrobial infections (83, 201). Based on the PCR detection of protein L sequences in *P. magnus* isolates, we conclude that protein L-containing isolates are rare. The only protein L-containing isolates detected all originated from Swedish BV patients and were not present in strains isolated from other infected patients from different countries. On the other hand, albumin-binding ($L^-$ $A^+$) isolates, while predominant in isolates from Sweden, were also found in one type strain and one isolate from Canada.

We used three different molecular typing methods (PCR-REA, ribotyping, and PFGE) to predict the relatedness of the $L^+$ $A^-$, $L^-A^+$, and $L^-A^-$ *P. magnus* isolates. All three molecular typing methods were successful in typing and were reproducible. The Simpson's index of discrimination aids in comparing typing systems and the level of discrimination depends on many factors where an index of greater than 0.90 is desireable (110). The PCR-REA of 16S rRNA sequences using six restriction endonucleases was not very discriminating with a *D* smaller than 0.5 (110). This is because 16S rRNA DNA sequences are conserved; therefore, as anticipated, isolates had little variation in RFLP within a well defined species. In other studies, PCR-REA has been shown to be moderately discriminatory because the gene targets were more variable. For example, PCR-REA of the spacer region between the 16S and 23S rRNA genes of 9 *Rochalimaea henselae* isolates were subtyped into 3 groups (157), PCR-REA with *Mse*I of an 820 base pair region of *ureC* for 25 *H. pylori* isolates resulted in 11 patterns (81), and PCR-REA with *Alu*I of a segment of the Staphylocoagulase gene of 30 *S. aureus*

isolates resulted in 10 patterns (87).

Short repetitive DNA sequences exist in the bacterial genome (e.g. REP (repetitive extragenic palindrome in members of the enterobacteriaceae) and ERIC (enterobacterial repetitive intergenic consensus)) which may be used to design PCR primers (rep-PCR) (148). These sequences are located throughout the genome, are found in intercistronic regions, and are transcribed but not translated (120). The functions of REPs or ERICs are unknown but may be involved in transcription termination, mRNA stability (the central inverted repeat allows the formation of stem-loop structures in transcribed RNA), or chromosomal organization (120, 148). Also, in arbitrarily primed PCR (AP-PCR), a single, short (usually 10 base pairs) primer, whose nucleotide sequence is not based on a known genetic locus is used (156). The use of arbitrary primers results in the amplification of one or more unpredictable loci producing a set of fragments (156). The advantages of AP-PCR include its simplicity and theoretically, it can be applied to any organism (8). However, there can be difficulties with AP-PCR in obtaining reproducible discriminating results: the low stringency conditions used for amplification often lead to individual bands with various intensities (8). Rep-PCR and arbitrarily primed PCR may be worth evaluating in future applications for typing *Peptostreptococcus* spp.

Because the whole genome is available for analysis, PFGE analysis produces greater discrimination than ribotyping (8, 141). The higher discriminatory power of PFGE over ribotyping with some bacteria has been previously reported: group B streptococci (89), *S. aureus* (225), *P. aeruginosa* (221), *E. faecalis* (90), *C. difficile* (129), and *L. pneumophila* (246). However, ribotyping is less expensive and time-consuming and does not require specialized equipment compared to other genotyping methods such as PFGE (156). Disadvantages of ribotyping include limited resolving power especially if the bacterial species has small numbers of *rrn* operons in their

genome (*Mycoplasma* spp., *Mycobacterium* spp., and *Borrelia burgdorferi* have only one *rrn* operon), and focuses on only one cluster of genes (8, 293). An advantage of PFGE is that banding patterns from PFGE gels are usually easy and clear for visualization (89). The ultimate strength of PFGE may be in its ability to confirm actual identity of isolates (141). Some disadvantages of PFGE are that it is technically demanding, time-consuming (preparation of DNA plugs takes up to four days), requires specialized equipment, and in certain cases there may be difficulties in achieving band identity reproducibility in different runs to assign observed patterns to types with full confidence (156, 242).

In this study, the discriminatory power of PFGE and the combined data ($D = 0.9341$) was greater than ribotyping ($D = 0.9231$). The three $L^+ A^-$ isolates were distinguished by PFGE and using the 3 typing schemes combined, were distinguished into individual banding pattern groups (V, VI and VII) whereas in ribotyping, the three $L^+ A^-$ isolates could only be distinguished into two banding pattern groups. However, for groups V, VI and VII, the Dice coefficients, which were based on PFGE and ribotyping results, ranged from 73.9% to 94.3% similarity, indicating that banding pattern differences in these groups were minor. Also, despite differences between them, the $L^+ A^-$ isolates were more closely related to each other than to the other isolates.

The interpretation of DNA band sizes for the 3 genotyping results (PCR-REA, ribotyping, and PFGE) was empirically decided using measurements ($R_f$ values) from the bottom of the agarose wells to the bottom of the DNA bands in comparison to DNA markers, comparing replicates for relative migration of DNA bands, and using a precision allowance of ± 1 mm as a guide (Fourney, personal communication).

The criteria for interpreting closely related strains are more conservative than previously

suggested criteria for PFGE alone (277). Since there is no previously established criteria established for cluster analysis of *P. magnus*, we chose 70% similarity as the break point for strain clustering. These criteria included isolates with less than 5 band differences using combined ribotyping and PFGE results. This criteria for categories of genetic and epidemiologic relatedness of isolates has been defined for pulsed-field gel electrophoresis analysis (277). Indistinguishable isolates have the same number of sized bands in their restriction patterns. These isolates are the same strain. Closely related isolates may vary in its restriction pattern by one single genetic event when compared to an outbreak reference pattern, i.e., a point mutation or an insertion or deletion resulting in 2 to 3 band differences. Possibly related isolates may vary in its restriction patterns by 2 independent genetic events when compared to an outbreak reference pattern (4 to 6 band differences). Unrelated isolates compared to the restriction pattern of the outbreak reference pattern have 3 or more independent genetic events (7 bands or more differences) (277).

With our criteria, 3 clusters of isolates were observed, the protein L-containing isolates and the 2 groups of Swedish $L^-$ $A^+$ isolates (Figure 9-12), with the rest of the isolates being unrelated. These clusters contained isolates that we consider clonal because the similarity within the clusters was much higher than between clusters or between genetically unrelated isolates (277). These clusters, which were all Swedish in origin, probably were derived from a common parent, even though the isolates were reported to be epidemiologically unrelated. This type of clustering analysis should be a useful approach for analyzing larger numbers of isolates to determine their relatedness or to identify strains from outbreaks. With the inclusion of more strains in the analysis, the interpretive cut-off for similarity might be decreased from 70%.

Alternative distance matrix methods such as the Fitch and Margoliash method (75) and the

Neighbour-joining method (238) are less sensitive to unequal rates of evolution (195). However, the UPGMA method was an effective method in clustering particular genotypes of *P. magnus* isolates (protein L-containing and albumin-binding) because the isolates that made up the clusters were so much more similar to one another than to isolates outside their clusters. Thus, if alternative algorithms other than the UPGMA method were used, the same cluster of isolates would most likely occur.

With the availability of automated DNA sequence techniques and PCR, nucleotide sequencing of the PCR product is now becoming a practical method which allows for comparison of numerous isolates by sequencing each one at the same locus (8). The target is generally a locus where enough sequence variability is present to allow for strain differentiation and must be present in all isolates examined (19). Some typed targets include the outer membrane porin, protein PIB gene of *N. gonorrhoeae* (140), and the major outer membrane protein of *C. trachomatis* (208). Although sequencing of the 16S rRNA genes from various *Peptostreptococcus* species has been performed for taxonomic purposes (143), it may be useful to sequence the variable regions of *rrn* operons for future applications as a typing method. Some advantages of nucleotide sequencing include precise data and the availability of data bases to be shared to allow for comparative analysis (8).

# 5. CONCLUSIONS AND SIGNIFICANCE

There are several important conclusions found in my studies:

1) The first hypothesis stated that the proteolytic activity of *Peptostreptococcus* isolates is due to proteinases and not nonspecific proteases such as aminopeptidases. There is no indication that *Peptostreptococcus* species contain proteinase genes after the construction of 2 genomic libraries of *P. magnus* Gifu 7723 in *E. coli* based vectors and screening for such activities on agar plate assays and with heterologus proteinase gene probes.

2) The second hypothesis stated that the proteolytic activity of *Peptostreptococcus* isolates is due to positive aminopeptidase reactions. The proteolytic activities of *Peptostreptococcus* species are due in part to aminopeptidase reactions based on weak activity on nonspecific and general substrates, proteolytic profiles, and statistical analysis. The equality of proportions for more than 2 samples indicated certain positive aminopeptidase reactions present in each of the 5 *Peptostreptococcus* species ($Z > 1.96$) were significant.

3) The third hypothesis states that aminopeptidase profiles are a reliable method to classify *Peptostreptococcus* species as compared to the VPI method. The species "*P. prevotii*" as defined by the "gold" standard method is a collection of heterogeneous isolates as confirmed by the pre-formed enzyme profiles of rapid ID32A and requires further taxonomic classification. Identification to the species level is important because it may lead to a better understanding of the main role for certain species in polymicrobial anaerobic infections. Certain *P. magnus* and *P. micros* isolates from the female genital tract could not be

distinguished from one another using the rapid ID32A kit. This could be a reflection of the site of isolation for these isolates or that this could reflect in a need for a new "gold" standard method. Therefore, aminopeptidase profiles may be useful for classification for particular *Peptostreptococcus* species.

4) The fourth hypothesis stated that protein L-containing and albumin-binding *P. magnus* isolates have a clonal structure. Protein L-containing and albumin-binding *P. magnus* isolates have a clonal structure and may be used as markers for identifying specific strain types in anaerobic infections including the vagina.

5) The fifth hypothesis stated that PFGE is the best genotyping method compared to PCR-REA and ribotyping and UPGMA is an effective method for clustering particular phenotypes of *P. magnus* isolates. PFGE and ribotyping are highly discriminating typing methods for *Peptostreptococcus magnus* strains. Cluster analysis using the UPGMA method is an effective method for clustering *Peptostreptococcus* magnus isolates based on certain phenotypes ($L^+$ or $A^+$).

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# 7. APPENDICES

## APPENDIX A.

## A. CONSTRUCTION OF GENOMIC LIBRARIES.

In this thesis, preliminary work on the cloning of proteolytic enzyme genes from peptostreptococci is reported as supplementary work. This includes the construction of genomic libraires of *P. magnus* Gifu 7723 (Appendix B, Table 20, Study 5), an ovarian abscess isolate, in *E. coli* based cloning vectors and screening for a proteinase gene using agar plate assays and heterologous DNA protease probes. As well, a genomic library of *P. anaerobius* ATCC 27337 (Appendix B, Table 20, Study 6) was constructed in the λZAP™ vector to screen for a proline aminopeptidase gene using chromogenic substrates and heterologous oligonucleotide probes.

### A.1 MATERIALS AND METHODS.

#### A.1.1 BACTERIAL ISOLATES.

Genomic libraries of *P. magnus* Gifu 7723, an ovarian abscess strain (70), and *P. anaerobius* ATCC 27337 were constructed. Dot blot hybridization studies of genomic DNA from 58 *Peptostreptococcus* isolates associated with the female genital tract after probing with the C5a peptidase gene included 7 *P. anaerobius*, 9 *P. asaccharolyticus*, 23 *P. magnus*, 10 *P. micros*, 7 *P. prevotii*, and 2 *P. productus* (Appendix B, Table 20, Study 4).

*E. coli* host strains and containing plasmids are listed (Appendix B, Table 21). The following recombinant plasmids containing proteolytic genes were used to obtain heterologous DNA probes: pYS307, *tpr* gene (cysteine protease) from *P. gingivalis* W83 on a 1.9 kb *Hin*cII-*Hin*dIII fragment (211); pSA100, *pro*A gene (metalloproteinase) from *L. pneumophila* on a 4.2 kb *Bam*HI-*Sal*I

fragment (24); pJG1, IgA gene (IgA protease) from *Streptococcus sanguis* on a 2.4 kb *Bam*HI-*Apa*I fragment (86); and pTT45, *scp*A gene (C5a peptidase) from *Streptococcus pyogenes* (43).

**A.1.2 GROWTH CONDITIONS.**

See section 2.2 for the growth conditions of *Peptostreptococcus* isolates. *E. coli* strains were grown on Trypticase Soya Agar (TSA) (Difco, Detroit. MI.) supplemented with the appropriate selective agents at 37°C if appropriate. *N. gonorrhoeae* CH811 was grown on GC medium base (GCMB; Difo) with 1% modified defined supplement in 5% $CO_2$ at 37°C for 24 hr. *Lactobacillus helveticus* ATCC 11443 and *L. lactis* NCDO 763 were grown on TSA at 32°C.

**A.1.3 PLASMID PURIFICATION.**

Plasmid extraction from *E. coli* was performed using an upscaled/larger volume version of the alkaline extraction method for recombinant plasmids containing protease genes and recombinant clones from various genomic libraries (18). Overnight cultures in 50 ml LB broth with appropriate selective agents if required were harvested and resuspended in 2.5 ml GTE buffer (50 mM glucose; 25 mM Tris-HCl, pH 8.0; 10 mM EDTA) and lysozyme (2 mg/ml). Suspended cultures were placed on ice for 30 min. Five ml of 0.2 N NaOH; 1.0% SDS was added to the resuspended cells and placed on ice for 5 min.; then, 3.75 ml of 5 M potassium acetate, pH 4.5 was added to the lysate and was placed on ice for 1 hr. The lysate was centrifuged at 12,500 rpm for 15 min. The supernatant was collected and precipitated with 2 volumes of ethanol at - 20°C overnight. DNA was spun down at 10,500 rpm for 10 min. Plasmid DNA was purified from chromosomal DNA using cesium chloride/ethidium-bromide gradients (239). Each quick-seal™ ultracentrifuge tube (Beckman Instruments Inc., Palo Alto, CA.) contained 3.3 ml of TE buffer and 2.4 g of cesium chloride with ethidium bromide (243 µg/ml). Tubes were spun down at 100,000 rpm at 10°C for 4 hr using the

benchtop Optima™ TLX ultracentrifuge (Beckman). DNA was collected in disposable tubes using a 5-gauge hypodermic needle and ethidium bromide was removed by adding an equal volume of water saturated iso-butanol and gently mixed to remove the upper layer. This step was repeated up to four times. The DNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 4.5) and 2 volumes of 95% ethanol at - 20°C overnight. The DNA was centrifuged at 10,000 rpm for 20 min. and resuspended in 100 µl of $ddH_2O$.

**A.1.4 GENOMIC DNA PURIFICATION.**

See section 2.8 for genomic DNA preparation of *Peptostreptococcus* species used for hybridization work and construction of genomic libraries. Extraction of genomic DNA from *E. coli* XL1-Blue MRF' and *N. gonorrhoeae* CH811 was performed as described previously (202). The DNA from *N. gonorrhoeae* CH811 was used for dot blot hybridization work. The DNA from *E. coli* XL1-Blue MRF' was used for slot blot hybridizations probed with various oligonucleotides designed from proline aminopeptidase gene sequences. Cells were grown overnight in 10 ml LB broth at 37°C and spun down. Cells were suspended in 3 ml TE buffer (10 mM Tris, pH 8.1; 1 mM EDTA) with 25% sucrose. Lysozyme was added at a final concentration of 1 mg/ml and the mixture was left on ice for 4 min. One hundred and sixty µl of 10% SDS was added to give a final concentration of 0.5%. The mixture was left at room temperature after gentle mixing until a clear lysate was seen. To the cleared lysate, 800 µl of 5 M sodium perchlorate was added to give a final concentration of 1 M and mixed gently at room temperature. An equal volume of a chloroform/isoamyl alcohol mixture (24:1 (v/v)) was added to the cleared lysate, mixed gently, and centrifuged at 10,000 rpm for 10 min. at 4°C. This was repeated until no visible debris was observed at the interface. Two volumes of 95% ethanol were added to the aqueous phase, mixed, and the DNA was precipitated for 1 hr at -20°C.

The DNA pellet was spun down at 10,000 rpm at 4°C , washed with 70% ethanol, and eventually resuspended in 1 ml TE buffer.

Genomic DNA extraction of *L. delbrueckii* ATCC 11443 and *L. lactis* NCDO 763 was performed using the following method (11). The genomic DNA was used for slot blot hybridizations probed with various oligonucleotides designed from proline aminopeptidase genes. Cells were grown overnight in 400 ml cultures of Trypticase Soy broth and resuspended in TEN buffer (10 mM Tris-HCl, pH 7.6; 1 mM EDTA; 10 mM NaCl). Mutanolysin (10 µg/ml) and lysozyme (2 mg/ml) were added for 2 hr at 37°C. SDS (0.5%) was added to lyse cells and proteins were digested with proteinase K at 37°C (0.2 mg/ml) overnight with gentle shaking. Deproteinization was carried out with multiple phenol and chloroform extractions. The DNA was precipitated with 95% ethanol overnight at - 20°C. Genomic DNA samples were further purified using cesium chloride/ethidium bromide gradients (239). The DNA was resuspended in 100 µl $ddH_2O$.

## A.1.5 CONSTRUCTION OF GENOMIC LIBRARIES OF *PEPTOSTREPTOCOCCUS* ISOLATES.

Partial *Sau*3A1 digests of genomic DNA (2-5 kb) from *P. magnus* Gifu 7723 were collected from sucrose density centrifugation gradients, and then ligated to *Bam*HI-digested and dephosphorylated phagemid vectors pBluescript KS (-) and pTZ18R. Preparation of competent *E. coli* DH5α cells for transformation was performed using the method of Chung *et al.* (50). An overnight culture of cells was diluted 1:100 into prewarmed LB broth and cells were incubated at 37°C with shaking at 225 rpm to reach an $OD_{600}$ of 0.3-0.4. An equal volume of ice-cold 2 X TSS ( LB broth with 5% PEG 8000; 2.5% dimethyl sulphoxide; and 50 mM $Mg^{2+}$ ($MgCl_2$)), pH 6.5 was added. For transformation, 0.1 ml aliquot of cells were pipetted into a cold polypropylene tube

containing 1 µl (100 pg) of plasmid DNA and the cell/DNA suspension was mixed gently and incubated at 4°C for 5-60 min. A 0.9 ml aliquot of LB broth plus 20 mM glucose was added and the cells were incubated at 37°C with shaking (225 rpm) for 1 hr to allow for expression of the selective gene. Six thousand recombinant clones of pBluescript KS (-) and 2,500 recombinant clones of pTZ18R were replica plated on 1% skim milk/Ap/X-gal agar plates. Screening for proteolytic activity involved two sets of growth conditions: 1) 1 day aerobically and then 3 days anaerobically (29) and, 2) 1 day anaerobically; 1 hr exposure to chloroform; and 2 weeks anaerobically (211).

*Sau*3AI partial genomic DNA digests of *P. anaerobius* ATCC 27337 that ranged in size from 8-10 kb were ligated into the λZAP express™ *Bam*HI/calf intestinal alkaline phosphatase (CIAP) vector and were transfected into *E. coli* XL1-Blue MRF' on NZY/Tetracycline plates according to the predigested λZAP express™ *Bam*HI/CIAP vector cloning kit manual (Stratagene). The λZap express™ vector contains the phagemid pBK-CMV and recombinant phagemids were excised with the ExAssist™ helper phage (M13) onto LB/Kanamycin (Km) plates. Five thousand recombinant clones were screened for proline aminopeptidase activity by flooding LB/Km replica plates with 0.2 ml proline β-naphthylamide (10 mg/ml in dimethylformamide) (Sigma, P-1380), 0.1 ml Fast Garnet GBC (10 mg/ml in dd$H_2$0) (Sigma F-8761), and 5 ml of 50 mM sodium phosphate buffer (pH 7.0) (41) under aerobic and anaerobic growth conditions (211).

**A.1.6 HYBRIDIZATION STUDIES WITH PROTEASE GENE PROBES.**

Plasmids were digested with the indicated restriction endonucleases, as required, and the fragment was isolated from a 0.8% low melting point agarose gel (Seaplaque, FMC Corporation). All gene probes were labelled nonradiometrically by the random primed DNA labelling method with digoxigenin using the Genius kit (Boehringer Mannheim Corporation). For 50 µl of probe, 38 µl of

denatured probe in $ddH_2O$ was mixed with 5 µl of random hexanucleotide primers and 5 µl of deoxyribose nucleotides/digoxigenin labelled dUTP solutions (10 mM dATP, dCTP, dGTP, 6.5 mM dTTP, and 3.5 mM digoxigenin-11-dUTP), and 2 µl Klenow polymerase enzyme. The mixture was labelled overnight at 37°C. Hybridizations were performed at low stringency (42°C in 5 X SSC, 0.02% SDS) with washes in 0.1 X SSC, 0.1% SDS. The detection procedure was at 25°C and included washing the filters for 1 min. with buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5). They were then incubated with buffer 1 with 0.5% blocking reagent for 30 min. The filters were washed again for 1 min. in buffer 1 and then incubated with diluted antibody-conjugate solution (anti-digoxigenin antibody at 1:5000 in buffer 1) for 30 min. The unbound antibody-conjugate was removed by washing with buffer 1 for 2 X 15 min. The filters were equilibrated for 2 min. with buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) and were incubated in the dark with freshly prepared colour solution (Nitroblue tetrazolium chloride (NBT) and X-phosphate solution (5-bromo-4-chloro-3-indolylphosphate in buffer 3). The detection reaction took 1-2 days and was stopped by washing in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Genomic DNA ($10^{-1}$ to $10^{-4}$ µg) from *P. magnus* Gifu 7723 were used to dot nylon membranes. One µg of genomic DNA for each of 58 *Peptostreptococcus* isolates was used for experiments with pTT45 (containing C5a peptidase gene) according to the Bio- Dot SF blotting apparatus protocol (Bio-Rad Laboratories, Richmond, CA.). The nylon membrane (Boehringer Mannheim Corporation) was pre-wet in 2 X SSC and the DNA samples were denatured to give a final concentration of 0.4 M NaOH, 10 mM EDTA and heated to 100°C for 10 min. to guarantee complete denaturation. The solution was neutralized by adding an equal volume of cold 2 M sodium acetate, pH 7.0. The DNA solution was then added to the Bio-Dot apparatus under vacuum. The nylon membrane was rinsed in 2 X SSC and air dried.

## A.1.7 SCREENING FOR A PROLINE AMINOPEPTIDASE GENE FROM *P. ANAEROBIUS* ATCC 27337 USING HETEROLOGOUS OLIGONUCLEOTIDE PROBES.

Alignment at the nucleotide level of 5 bacterial proline aminopeptidase genes was performed using PC Gene (Intelligenetics, Inc., Mountain View, CA.) and Blast searches (6). Three oligonucleotide probes (GP1, LB1, and LB2) were designed from the 5 bacterial proline aminopeptidase genes. The 18-mer oligonucleotide GP1 was from the *pap* gene of *B. coagulans* (Position 167 (5'-TTTATATGACCAATTGGG-3')) (121). The 18-mer oligonucleotide LB1 was from the *pepXP* gene of *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* (Position 465, respectively (5'- CAAATCGTTGGCAACCTT-3')) (159, 193). Finally, the 19-mer degenerate oligonucleotide LB2 was from the *pepIP* gene of *L. delbrueckii* subsp. *bulgaricus* and the *pep*PN gene of *L. helveticus* CNRZ32 (Positions 413 and 328, respectively (5' -CAAAGCTG-GGGCGG(G/A)C/AT(G/T)C-3')) (11, 60). All 3 oligonucleotides were labelled radiometrically with [$\alpha$ -$^{32}$]PdATP (Amersham) using the DNA tailing kit (Boehringer Mannheim Canada) and purified similar to the DIG oligonucleotide tailing kit. Slot blot hybridizations were performed accordingly (203). Two-fold dilutions of genomic DNA (0.2 µg/ml- 0.0125 µg/ml) and pSUW92 DNA (0.00625 µg/ml to 0.000390 µg/ml) were prepared. The hybridization solution consisted of 10% polyethylene glycol; 1.5 X SSPE; and 7.0% SDS with 250 µl herring sperm DNA (11 µg/ml) (Promega). The $T_m$ for each oligonucleotide was calculated using the formula $T_m = 4(G + C) + 2(A + T)$ and the hybridization temperature was determined using Tm-5 to 20°C as a guide (296). The $T_m$ was determined for the following oligonucleotides: GP1 (48°C), LB1 (52° C), and LB2 (66° C). The hybridization temperatures used for the three oligonucleotide probes were as follows: GP1 (28°C, 33°C, and 42°C); LB1 (41 °C and 46° C) and; LB2 (41° C and 46° C). Washes were performed as

follows: 2 X SSPE (15 min.); 2 X SSPE; 0.1% SDS (3 X 5 min.); and 0.1 X SSPE; 0.1% SDS (1 hr) at 28°C for GP1 and at 38°C for LP1 and LP2. X-ray films were exposed overnight at -70°C.

## A.2 RESULTS.

### A.2.1 SCREENING AND CONSTRUCTION OF GENOMIC LIBRARIES.

In earlier reports, it was shown that *P. magnus* isolates from soft tissue infections produced collagenases (127-128). A genomic library of *P. magnus* Gifu 7723 was constructed in pBlusecript KS (-) and pTZ18R to screen for proteinase activity. Screening of 6,000 recombinant clones of pBluescript KS (-) and 2,500 recombinant clones of pTZ18R under the two conditions resulted in no positive proteolytic clones on 1% skim milk/Ap/X-gal agar plates. *P. magnus* Gifu 7723 was used as the positive control.

### A.2.2 DOT BLOT HYBRIDIZATIONS OF *P. MAGNUS* GIFU 7723 PROBED WITH HETEROLOGOUS PROTEASE GENES.

In an attempt to identify a protease gene from *P. magnus* Gifu 7723, dot blot hybridizations of genomic DNA from *P. magnus* Gifu 7723 and other *Peptostreptococcus* isolates were probed with 4 heterologous protease genes (a cysteine proteinase from *P. gingivalis* W83, a metalloproteinase gene from *L. pneumophila*, the IgA protease gene from *S. sanguis* (Figure 14), and the C5a peptidase gene from *S. pyogenes*) showed no homology (Figure 15). In each case, the corresponding plasmid vector DNA (i.e. pSA110, pYS307, pJG1, and pTT45) that contained the corresponding proteolytic gene was used as the positive control.

### A.2.3 SCREENING FOR A PROLINE AMINOPEPTIDASE GENE IN *P. ANAEROBIUS* ATCC 27337.

*P. anaerobius* is positively correlated with BV (101) and may contribute to the elevated levels

of proline aminopeptidase in vaginal fluids of BV patients (248). The identification of the proline aminopeptidase gene(s) from *P. anaerobius* ATCC 27337 may be useful for diagnostic purposes in BV. The genomic library of *P. anaerobius* ATCC 27337 was constructed in the λZAP™ vector. Five thousand recombinant clones derived from pBK-CMV in *E. coli* showed no proline aminopeptidase activity with the chromogenic substrates proline β-naphthylamide and Fast Garnet GBC. Screening genomic DNA from *P. anaerobius* ATCC 27337 with heterologous proline aminopeptidase gene sequences showed no direct evidence of a proline aminopeptidase gene being present. With the oligonucleotides GP1, LB1, and LB2, there was no specific positive signals for a proline aminopeptidase gene of *P. anaerobius* ATCC 27337.

**Figure 13.** Dot blot hybridization of *P. magnus* Gifu 7723 genomic DNA using heterologous protease gene probes: **a)** *tpr* gene probe from *P. gingivalis* W83 (cysteine proteinase); **b)** *proA* gene probe from *L. pneumophila* (metalloproteinase); and **c)** IgA gene probe from *S. sanguis* (IgA protease).

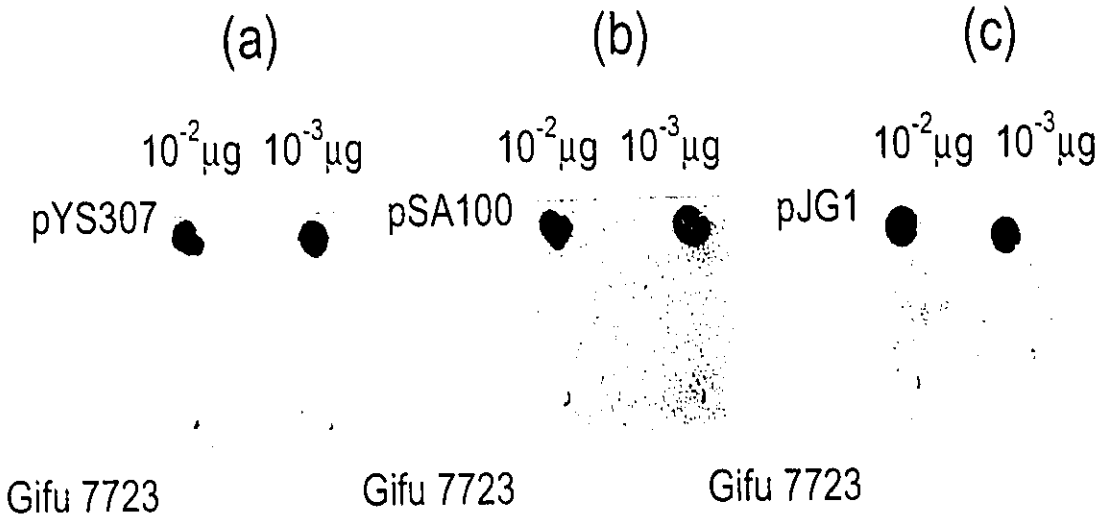
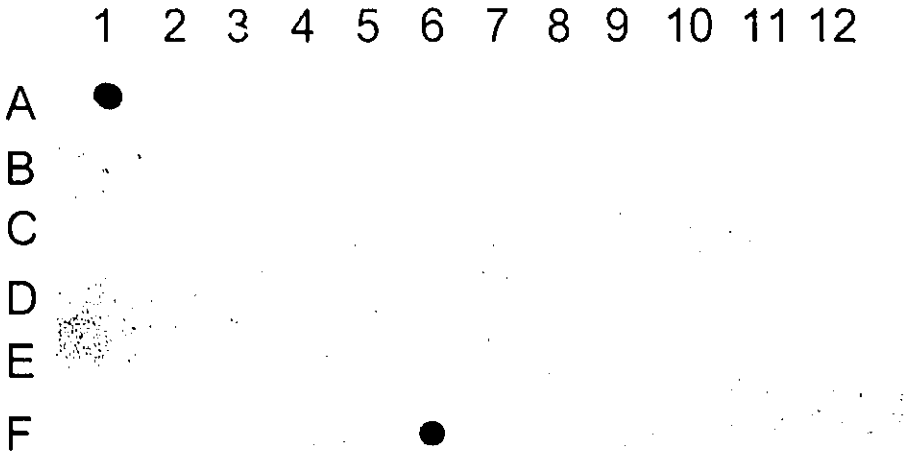

(a)
(b)
(c)
$10^{-2}$µg $10^{-3}$µg
$10^{-2}$µg $10^{-3}$µg
$10^{-2}$µg $10^{-3}$µg
pYS307
pSA100
pJG1
Gifu 7723
Gifu 7723
Gifu 7723

**Figure 14.** Dot blot hybridization of genomic DNA from 58 *Peptostreptococcus* isolates, including *P. magnus* Gifu 7723 probed with a C5a peptidase gene (A3). A1: pTT45, F6: pUC8, positive controls. A4: *N. gonorrhoeae* CH811, negative control. Other blots comprise the following *Peptostreptococcus* isolates: 22 *P. magnus* (A2, 5, 8, 10; B5, 10; C1, 8, 10-11; D8-12; E6, 8, 10-12; F1-2), 10 *P. micros* (A11; B6, 11; C2, 6, 12; E2, 7, 9; F3), 7 *P. anaerobius* (A6; B2-3, 10, 12; E4; F4), 9 *P. asaccharolyticus* (A7, 9, 12; B1; C9; D1, 3-4; F5), 7 *P. prevotii* (B7; C3; D2, 5-6; E3, 5), and 2 *P. productus* (C5, 7). All other wells were empty.


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F

## A.3 DISCUSSION.

### A.3.1 PHENOMENON OF PROTEOLYTIC ACTIVITY OBSERVED WITH *PEPTOSTREPTOCOCCUS MAGNUS* GIFU 7723 AND OTHER *PEPTOSTREPTOCCUS* ISOLATES.

Although a zone of hydrolysis was observed on skim milk agar plates after inoculation of *P. magnus* Gifu 7723 under anaerobic conditions after two or three days, this proteolytic activity may be due to cell lysis and death (250). The lack of proteinase activity observed with screening two genomic libraries and the lack of homology with screening genomic DNA with heterologous protease probes suggests that proteinase genes may not be present in *Peptostreptococcus* isolates used in these studies. Previous studies claimed that certain clinical isolates of *P. magnus* produced collagenases using the protocol of Steffen and Hentges, 1981 (127-128, 264). In this protocol, autoclaved collagen was mixed with the growth medium and inoculated with the appropriate cultures under anaerobic conditions for two weeks (127). However, proteolytic enzymes termed collagenases hydrolyzes the scission of peptide bonds located in the poly-L-proline of the helical regions when the substrate is undenatured (250). Enzymes are not collagenases if they hydrolyze native collagen in terminal regions of the chains outside the collagen fold, if they digest denatured collagen which is "parent gelatin" upon mild heating/autoclaving and with the addition of certain salts, or if they cleave oligopeptides that resemble sequences found in the helical regions of the α chains of collagen (250). This strongly suggests the proteolytic activity observed on collagen by these *P. magnus* isolates may be due to nonspecific proteases rather than collagenases. It is possible to observe a time-dependent (slower) degradation of autoclaved collagen with nonspecific proteases (160).

The lack of expression for proline aminopeptidase activity in the genomic library of *P.*

*anaerobius* ATCC 27337 and the lack of sequence homology of its genomic DNA to heterologous proline aminopeptidase genes is inconclusive as to whether it has a proline aminopeptidase gene. Proline aminopeptidase genes are heterogeneous in nature and may act on a variety of proline-containing substrates (240).This may explain the lack of homology seen. There could also be a screening, expression, and/or selection problems with proteolytic genes of *Peptostreptococcus* species in an *E. coli* background.

## APPENDIX B.

## B. BACTERIAL ISOLATES AND PLASMIDS.

**Table 20.** *Peptostreptococcus* isolates and strains.

| Genus and species | Strain or isolate | Origin of isolation | Supplier | Used in Rapid ID32A | Study |
|---|---|---|---|---|---|
| *P. anaerobius* | ATCC 27337[b] | Type strain | ATCC[a] | + | 1, 2, 3, and 6 |
| | CS1057A | Cesarian section | A. Chow | - | 4 |
| | CS1805 | Cesarian section | A. Chow | - | 4 |
| | CS2295A | Cesarian section | A. Chow | + | 1 and 2 |
| | CS2534 | Cesarian section | A. Chow | - | 4 |
| | MPC9793 | Mucopurulent cerevicitis | A. Chow | + | 1 and 2 |
| | MPC9804 | Mucopurulent cerevicitis | A. Chow | + | 1 and 2 |
| | PID3526F | Pelvic inflammatory disease | A. Chow | - | 4 |
| | PID3528E | Pelvic inflammatory disease | A. Chow | - | 4 |
| | SA533D2 | Septic abortion | A. Chow | + | 1 and 2 |
| | SA1733A | Septic abortion | A. Chow | + | 1 and 2 |
| | SA1789A | Septic abortion | A. Chow | + | 1 and 2 |
| | SA1894A | Septic abortion | A. Chow | + | 1 and 2 |
| | SA1895C | Septic abortion | A. Chow | + | 1 and 2 |
| | SA1929H | Septic abortion | A. Chow | + | 1 and 2 |
| | SA1960C | Septic abortion | A. Chow | + | 1 and 2 |
| | SA1972F | Septic abortion | A. Chow | + | 1 and 2 |
| | SA2820D | Septic abortion | A. Chow | + | 1 and 2 |
| | SA3018E | Septic abortion | A. Chow | + | 1 and 2 |
| | SA30185lg | Septic abortion | A. Chow | + | 1 and 2 |
| | T9605[b] | Tampon | A. Chow | - | 4 |
| | T9724 | Tampon | A. Chow | + | 1, 2, and 4 |
| | A18 | Unknown | P. Ewan | + | 1 and 2 |

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6- Construction of *P. anaerobius* ATCC 27337 genomic library.

**Table 20 continued.**

| Genus and species | Strain or isolate | Origin of isolation | Supplier | Used in Rapid ID32A | Study |
|---|---|---|---|---|---|
| | 781233 | Unknown | P. Ewan | + | 1 and 2 |
| | 1981-75 | Unknown | P. Ewan | + | 1 and 2 |
| | VH8970 | Vaginal hysterectomy | A. Chow | + | 1 and 2 |
| *P. asaccharolyticus* | ATCC 14963[b] | Type strain | ATCC | + | 1 and 2 |
| | CS1437A | Cesarian section | A. Chow | + | 1 and 2 |
| | CS1510C | Cesarian section | A. Chow | + | 1 and 2 |
| | CS1523D | Cesarian section | A. Chow | + | 1 and 2 |
| | CS1753E | Cesarian section | A. Chow | + | 1 and 2 |
| | CS1965 | Cesarian section | A. Chow | - | 4 |
| | CS2025A | Cesarian section | A. Chow | - | 4 |
| | CS2170C | Cesarian section | A. Chow | + | 1 and 2 |
| | CS2508C | Cesarian section | A. Chow | - | 4 |
| | MPC9802 | Mucopurulent cerevicitis | A. Chow | + | 1 and 2 |
| | MPC9818 | Mucopurulent cerevicitis | A. Chow | + | 1 and 2 |
| | MPC9820 | Mucopurulent cerevicitis | A. Chow | + | 1 and 2 |
| | PID992A | Pelvic inflammatory disease | A. Chow | + | 1 and 2 |
| | PID2008C | Pelvic inflammatory disease | A. Chow | + | 1 and 2 |
| | PID3442K | Pelvic inflammatory disease | A. Chow | + | 1 and 2 |
| | PID3485H | Pelvic inflammatory disease | A. Chow | + | 1 and 2 |
| | PID3615H | Pelvic inflammatory disease | A. Chow | + | 1, 2, and 4 |
| | PID3618F | Pelvic inflammatory disease | A. Chow | - | 4 |
| | SA508A | Septic abortion | A. Chow | + | 1 and 2 |
| | SA806C | Septic abortion | A. Chow | + | 1 and 2 |
| | SA1772D | Septic abortion | A. Chow | + | 1 and 2 |
| | SA2866D | Septic abortion | A. Chow | + | 1 and 2 |
| | T8127 | Tampon | A. Chow | - | 4 |
| | T8134 | Tampon | A. Chow | - | 4 |

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**Table 20 continued.**

| Genus and species | Strain or isolate | Origin of isolation | Supplier | Used in Rapid ID32A | Study |
|---|---|---|---|---|---|
| | T9676 | Tampon | A. Chow | + | 1, 2, and 4 |
| | T9826 | Tampon | A. Chow | - | 4 |
| | A133 | Unknown | P. Ewan | + | 1 and 2 |
| | AN2-413A | Unknown | P. Ewan | + | 1 and 2 |
| | AN2-419 | Unknown | P. Ewan | + | 1 and 2 |
| | AN2-434C | Unknown | P. Ewan | + | 1 and 2 |
| | 821758 | Unknown | P. Ewan | + | 1 and 2 |
| | 83-169 | Unknown | P. Ewan | + | 1 and 2 |
| *P. magnus* | ATCC 15794[b] | Type strain | ATCC | + | 1, 2, and 3 |
| | ATCC 53516 | Bacterial vaginosis | ATCC | + | 1, 2, 3, and 4 |
| | 564 | Bacterial vaginosis | E. Holst | - | 3 |
| | 595 | Bacterial vaginosis | E. Holst | - | 3 |
| | 624 | Bacterial vaginosis | E. Holst | - | 3 |
| | KA | Bacterial vaginosis | E. Holst | - | 3 |
| | BL 3431 | Breast abscess | E. Holst | - | 3 |
| | CS1966B | Cesarian section | A. Chow | + | 1, 2, 3, and 4 |
| | BL 1116 | Intraabdominal abscess | E. Holst | - | 3 |
| | MPC 9809 | Mucopurulent cervicitis | A. Chow | - | 4 |
| | MPC9814 | Mucopurulent cervicitis | A. Chow | - | 3 |
| | Gifu 7651 | Ovarian abscess | T. Ezaki | + | 1, 2, 3, and 4 |
| | Gifu 7723 | Ovarian abscess | T. Ezaki | + | 1, 2, 3, 4, and 5 |
| | BL 3410 | Pelvic abscess | E. Holst | - | 3 |
| | PID606D2 | Pelvic inflammatory disease | A. Chow | + | 1, 2, 3, and 4 |
| | PID3603 | Pelvic inflammatory disease | A. Chow | + | 1, 2, 3, and 4 |
| | BL 1730 | Scrotal abscess | E. Holst | - | 3 |
| | SA468A[b] | Septic abortion | A. Chow | + | 1 and 2 |
| | SA609A1 | Septic abortion | A. Chow | + | 1, 2 and 3 |
| | SA652A | Septic abortion | A. Chow | - | 3 and 4 |

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**Table 20 continued.**

| Genus and species | Strain or isolate | Origin of isolation | Supplier | Used in Rapid ID32A | Study |
|---|---|---|---|---|---|
| | SA1339F | Septic abortion | A. Chow | - | 3 and 4 |
| | SA1537[b] | Septic abortion | A. Chow | + | 1, 2, and 4 |
| | SA1734A | Septic abortion | A. Chow | + | 1, 2, 3, and 4 |
| | SA2005C | Septic abortion | A. Chow | - | 3 and 4 |
| | SA2065C | Septic abortion | A. Chow | + | 1 and 2 |
| | Gifu 7654 | Skin abscess | T. Ezaki | + | 1, 2, 3, and 4 |
| | Gifu 7735 | Skin abscess | T. Ezaki | + | 1, 2, 3, and 4 |
| | T8126[b] | Tampon | A. Chow | + | 1, 2, and 4 |
| | T8133 | Tampon | A. Chow | + | 1, 2, 3, and 4 |
| | T8150 | Tampon | A. Chow | - | 4 |
| | T8154 | Tampon | A. Chow | + | 1, 2, 3, and 4 |
| | T8156 | Tampon | A. Chow | + | 1, 2, 3, and 4 |
| | T9610 | Tampon | A. Chow | - | 3 and 4 |
| | T9664[b] | Tampon | A. Chow | + | 1, 2, and 4 |
| | A95 | Unknown | P. Ewan | + | 1, 2, and 3 |
| | AN2-970 | Unknown | P. Ewan | + | 1, 2, and 3 |
| | 791273 | Unknown | P. Ewan | + | 1, 2, and 3 |
| | VH8911[b] | Vaginal hysterectomy | A. Chow | + | 1, 2 and 4 |
| | VH8962[b] | Vaginal hysterectomy | A. Chow | + | 1 and 2 |
| | VH9072 | Vaginal hysterectomy | A. Chow | - | 3 and 4 |
| | BL 2771 | Wound | E. Holst | - | 3 |
| *P. micros* | ATCC 33270[b] | Type strain | ATCC | + | 1 and 2 |
| | BR4-7 | Breast abscess | C. E. Edmiston | + | 1 and 2 |
| | BR29-13 | Breast abscess | C. E. Edmiston | + | 1 and 2 |
| | BR48-7 | Breast abscess | C. E. Edmiston | + | 1 and 2 |
| | BR54-6 | Breast abscess | C. E. Edmiston | + | 1 and 2 |
| | BR55-3 | Breast abscess | C. E. Edmiston | + | 1 and 2 |
| | CS1509D | Cesarian section | A. Chow | + | 1 and 2 |
| | CS1952B | Cesarian section | A. Chow | - | 4 |

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**Table 20 continued.**

| Genus and species | Strain or isolate | Origin of isolation | Supplier | Used in Rapid ID32A | Study |
|---|---|---|---|---|---|
| | PID863A[b] | Pelvic inflammatory disease | A. Chow | + | 4 |
| | PID3430B[b] | Pelvic inflammatory disease | A. Chow | + | 1 and 2 |
| | SA838E[b] | Septic abortion | A. Chow | + | 1 and 2 |
| | SA2297E | Septic abortion | A. Chow | + | 1 and 2 |
| | SA3050[b] | Septic abortion | A. Chow | - | 4 |
| | T8079[b] | Tampon | A. Chow | - | 4 |
| | T9601 | Tampon | A. Chow | + | 1, 2, and 4 |
| | T9609[b] | Tampon | A. Chow | + | 1, 2, and 4 |
| | T9643[b] | Tampon | A. Chow | + | 1, 2, and 4 |
| | T9670 | Tampon | A. Chow | + | 1 and 2 |
| | T9833 | Tampon | A. Chow | - | 4 |
| | A53 | Unknown | P. Ewan | + | 1 and 2 |
| | A120 | Unknown | P. Ewan | + | 1 and 2 |
| | VH8898[b] | Vaginal hysterectomy | A. Chow | + | 1, 2, and 4 |
| | VH8952[b] | Vaginal hysterectomy | A. Chow | + | 1 and 2 |
| | VH9006[b] | Vaginal hysterectomy | A. Chow | + | 1 and 2 |
| | VH9074 | Vaginal hysterectomy | A. Chow | - | 4 |
| *P. prevotii* | ATCC 9321[b] | Type strain | ATCC | + | 1 and 2 |
| | CS1519B | Cesarian section | A. Chow | - | 4 |
| | CS2620C[b] | Cesarian section | A. Chow | + | 1, 2, and 4 |
| | PID676H[b] | Pelvic inflammatory disease | A. Chow | + | 1 and 2 |
| | PID936J[b] | Pelvic inflammatory disease | A. Chow | + | 1 and 2 |
| | PID990B[b] | Pelvic inflammatory disease | A. Chow | + | 1 and 2 |
| | PID3401G[b] | Pelvic inflammatory disease | A. Chow | + | 1, 2, and 4 |
| | SA838A[b] | Septic abortion | A. Chow | - | 4 |
| | SA1171F1 | Septic abortion | A. Chow | - | 4 |
| | SA2034A[b] | Septic abortion | A. Chow | - | 4 |

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6- Construction of *P. anaerobius* ATCC 27337 genomic library.

**Table 20 continued.**

| Genus and species | Strain or isolate | Origin of isolation | Supplier | Used in Rapid iD32A | Study |
|---|---|---|---|---|---|
| | A129[b] | Unknown | P. Ewan | + | 1 and 2 |
| | 83-125[b] | Unknown | P. Ewan | + | 1 and 2 |
| | 801478[b] | Unknown | P. Ewan | + | 1 and 2 |
| | 811556[b] | Unknown | P. Ewan | + | 1 and 2 |
| | 831833[b] | Unknown | P. Ewan | + | 1 and 2 |
| | AN2-253[b] | Unknown | P. Ewan | + | 1 and 2 |
| | VH9078 | Vaginal hysterectomy | A. Chow | - | 4 |
| *P. productus* | VH8958opague | Vaginal hysterectomy | A. Chow | | 4 |
| | VH8958large | Vaginal hysterectomy | A. Chow | - | 4 |

[a] American Type Culture Collection.
[b] Reconfirmation of identity by Dr. Shupeng Chou.
1- Identified by the rapid ID32A kit.
2- Aminopeptidase screening and gelatin hydrolysis.
3- Molecular epidemiology study.
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**Table 21.** *E. coli* strains and plasmids.

| Host strain | Genotype | Protease-containing plasmids and plasmid vectors | Supplier (Reference) |
|---|---|---|---|
| DH5α | *supE*44 Δ*lac*U169 (ϕ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1 | pSA100; *pro*A gene (metallo-protease) from *L. pneumophila.* | Dr. L. Tompkins (Dept. Microbiology and Immunology), Stanford University, Stanford CA. (24) |
| | | pYS307; *tpr* gene (cysteine protease) from *P. gingivalis* W83. | Dr. B. C. McBride (Departments of Microbiology and Oral Biology), University of British Columbia, British Columbia, Canada (206). |
| | | pTT45; *scp*A gene (C5a peptidase) from *S. pyogenes.* | Dr. P. P. Cleary (Department of Microbiology), University of Minnesota, Minneapolis, MN (43). |
| | | pSUW92; *pep*PN gene from *L. helveticus* CNRZ32. | Dr. J. L. Steele (Department of Food Science), University of Wisconsin-Madison, Madison, WI (60). |
| MM294 | *supE*44 *hsdR endA*1 *pro thi* | pJG1; IgA gene (IgA protease) from *S. sanguis.* | Dr. A. G. Plaut (Department of Molecular Biology and Microbiology) Tufts University, Boston, MA (84). |
| JM83 | ara Δ(*lac-proAB*) *rpsL* (ϕ80*lacZ*ΔM15) | pBluescript KS (-)<br>pTZ18R.<br>pUC8 | Stratagene, LaJolla, CA.<br>(164) |

**Table 21 continued.**

| Host strain | Genotype | Protease-containing plasmids and plasmid vectors | Supplier (Reference) |
|---|---|---|---|
| XL1-Blue MRF' | Δ (*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA*1 *supE*44 *thi*-1 *recA*1 *gyrA*96 *relA*1 *lac* [F' *proAB lacI*$^{q}$ZΔM15 Tn*10* (tet$^{r}$)]) | λZAP express™ | Stratagene, LaJolla, CA. |
| XLOLR | Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA*1 *thi*-1 *recA*1 *gyrA*96 *relA*1 *lac* [F' *proAB lacI*$^{q}$ZΔM15 Tn*10* (tet$^{r}$)] λ$^{r}$ Su$^{-}$ (nonsuppressing) | | Stratagene, LaJolla, CA. |

## APPENDIX C.

## STATISTICAL ANALYSIS TABLES.

**Table 22.** Determination of significant aminopeptidase reactions associated with 5 *Peptostrepiococcus* species using the equality of proportion analysis.

| Species (no. of isolates) | % isolates hydrolyzing gelatin | No. of profiles | Aminopeptidase reactions [a, b, c] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | ARG[d] | PRO | LG[d] | PHE | LEU[d] | PYR[d] | TYR[d] | ALA[d] | GLY[d] | HIS[d] | GG | SER[d] |
| *P. anaerobius* ($n = 20$) | 10.0 | 5 | - | + | - | - | - | - | - | - | - | - | - | - |
| *P. asaccharolyticus* ($n = 25$) | 8.0 | 9 | + | - | - | - | + | - | + | - | + | + | + | - |
| *P. magnus* ($n = 24$) | 95.8 | 9 | + | - | + | - | + | + | + | + | + | + | - | + |
| *P. micros* ($n = 19$) | 78.9 | 11 | + | + | + | + | + | + | + | + | + | + | + | + |
| *P. prevotii* ($n = 12$) | 16.7 | 12 | + | - | + | - | + | + | + | + | + | + | + | + |

[a] + means significant/ - means insignificant; Equality of proportions for more than two samples; $Z > 1.96$.

[b] See Table 4 for abbreviations of aminopeptidase reactions.

[c] Identified by the Rapid ID32A kit.

[d] $\chi^2$ analysis; $P < 0.001$.

$H_o$: The presence of certain positive aminopeptidase reactions among the 5 *Peptostreptococcus* species are not significant.

**Table 23.** Chi-square analysis between gelatin hydrolysis and *Peptostreptococcus* species.

| Gelatin Hydrolysis | *Peptostreptococcus* species (Expected No.) | | | | | Total # Isolates |
|---|---|---|---|---|---|---|
| | *P. anaerobius* | *P. asaccharolyticus* | *P. magnus* | *P. micros* | *P. prevotii* | |
| + | 2 (8.8) | 2 (11) | 23 (10.56) | 15 (8.36) | 2 (5.28) | 44 |
| - | 18 (11.2) | 23 (14) | 1 (13.44) | 4 (10.64) | 10 (6.72) | 56 |
| No. Isolates | 20 | 25 | 24 | 19 | 12 | 100 |

$\chi^2 = \sum_{\text{all } ij} \frac{(O_{ij} - E_{ij}^2)}{E_{ij}}$; E - expected frequency; O - observed frequency; $E_{ij} = \frac{\text{(total for row } i\text{)(total for column } j\text{)}}{\text{grand total}}$

$$\chi^2 = \frac{(2-8.8)^2}{8.8} + \frac{(2-11.0)^2}{11.0} + \frac{(23.0-10.56)^2}{10.56} + \frac{(15.0-8.36)^2}{8.36} + \frac{(2.0-5.28)^2}{5.28} + \frac{(18\ 0-11.2)^2}{11.2} + \frac{(23.0-14.0)^2}{14.0} + \frac{(1.0-13.44)^2}{13.44} + \frac{(4.0-10.64)^2}{10.64} + \frac{(10.0-6.72)^2}{6.72}$$

$= 61.74$

$\chi^2 > 18.47$

$df = (2-1)(5-1)$

$= 4$

$P < 0.001$

$H_o$: There is no association between gelatin hydrolysis and *Peptostreptococcus* species.

**Table 24.** Table for determining the linear correlation coefficient[a] of the data % gelatin hydrolysis vs the number of aminopeptidase substrates hydrolyzed for 100 *Peptostreptococcus* isolates.

| Gelatinase Activity | Number of aminopeptidase reactions[b] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| + | 2 | 0 | 1 | 3 | 1 | 8 | 4 | 7 | 10 | 2 | 1 | 5 |
| - | 14 | 4 | 7 | 8 | 10 | 1 | 5 | 3 | 3 | 2 | 0 | 2 |
| %[c] | 12.5 | 0.0 | 12.5 | 37.5 | 9.1 | 88.8 | 44.4 | 70.0 | 76.9 | 50.0 | 100.0 | 71.4 |
| $y$ [d,e] | 6.49 | 14.0 | 21.5 | 29.0 | 36.5 | 44.0 | 51.5 | 59.0 | 66.5 | 74.0 | 81.5 | 89.0 |
| $y^{RESID}$[f] | 6.00 | -14.0 | -9.0 | 8.5 | -27.4 | 44.8 | -7.1 | 10.9 | 10.4 | -24.0 | 18.5 | -17.6 |

[a] $\sum_{j=1}^{N}(X_i-\bar{X})(Y_i-\bar{Y})$ (See Materials and methods)

$$r = \frac{}{\sqrt{\sum_{j=1}^{N}(X-\bar{X})^2\,(Y-\bar{Y})^2}}$$

[b] x-axis; [c] y-axis

[d] $\hat{y} = b_o + b_1x$; $b_o = 1/n\,(\sum_{j=1}^{N}y - b_1\sum_{j=1}^{N}x)$; $b_1 = S_{XY}/S_{XX}$; $S_{XY} = \sum_{j=1}^{N}(x-\bar{x})^2$; $S_{XX} = \sum_{j=1}^{N}(x-\bar{x})\,(y-\bar{y})$

[e] $\hat{y} = -1.007576 + 7.502488x$

[f] $y^{RESID} = y-\hat{y}$

(Residual = Observed - Predicted)

**Table 25.** Chi-square analysis between gelatin hydrolysis and the number of aminopeptidase substrates hydrolyzed.

| Gelatin hydrolysis | Number of positive aminopeptidase reactions (Expected No.) | | No. isolates |
|---|---|---|---|
| | 1-5 | 6-12 | |
| - | 7 (20.7) | 37 (23.3) | 44 |
| + | 40 (26.3) | 16 (29.7) | 56 |
| No. isolates | 47 | 53 | 100 |

$\chi^2 = \sum_{all\ ij} (O_{ij} - E_{ij})^2 / E_{ij}$; E - expected frequency; O - observed frequency; $E_{ij} = \frac{\text{(total for row } i\text{)(total for column } j\text{)}}{\text{grand total}}$

$$\chi^2 = \frac{(7.0-20.7)^2}{20.7} + \frac{(37.0-23.3)^2}{23.3} + \frac{(40.0-26.3)^2}{26.3} + \frac{(16.0-29.7)^2}{29.7}$$

$= 30.58$
$\chi^2 > 10.83$
$df = (2-1)(2-1)$
$= 1$
$P < 0.001$

$H_o$: There is no association between % gelatin hydrolyzing isolates and the number of positive aminopeptidase reactions as arbitrarily defined.

**Table 26.** Distribution of aminopeptidase reactions for both gelatin positive and negative isolates among the 5 *Peptostreptococcus* species.

| Aminopeptidase reactions [a, b, c] | Gelatin Hydrolysis | Species | | | | | |
|---|---|---|---|---|---|---|---|
| | | *P. anaerobius* | *P. assacharolyticus* | *P. magnus* | *P. micros* | *P. prevotii* | Total |
| ARG | + | 0 | *2[c]* | *23[c]* | *15[c]* | *2[c]* | 42 |
| | - | 1 | *23* | *1* | *4* | *10* | 39 |
| PRO | + | *2[c]* | 0 | 0 | *8[c]* | 1 | 11 |
| | - | *18* | 0 | 1 | *3* | 0 | 22 |
| LG | + | 0 | 0 | *20[c]* | *14[c]* | *1[c]* | 35 |
| | - | 0 | 0 | *1* | *2* | *5* | 8 |
| LEU | + | 0 | *2[c]* | *23[c]* | *15[c]* | *2[c]* | 42 |
| | - | 1 | *16* | *1* | *4* | *8* | 30 |
| PYR | + | 0 | 0 | *23[c]* | *15[c]* | *1[c]* | 39 |
| | - | 0 | 0 | *1* | *3* | *5* | 9 |
| TYR | + | 0 | *1[c]* | *10[c]* | *10[c]* | *1[c]* | 22 |
| | - | 0 | *21* | *1* | *4* | *5* | 31 |
| ALA | + | 0 | 0 | *20[c]* | *13[c]* | *2[c]* | 35 |
| | - | 1 | 2 | *1* | *4* | *6* | 14 |

**Table 26 continued.**

| Aminopeptidase reactions [a, b, c] | Gelatin Hydrolysis | Species | | | | | Total |
|---|---|---|---|---|---|---|---|
| | | *P. anaerobius* | *P. assacharolyticus* | *P. magnus* | *P. micros* | *P. prevotii* | |
| GLY | + | 0 | *0*[c] | *22*[c] | *13*[c] | *2*[c] | 37 |
| | - | 1 | *11* | *1* | *3* | *9* | 25 |
| HIS | + | 0 | *2*[c] | *13*[c] | *13*[c] | *1*[c] | 29 |
| | - | 0 | *23* | *1* | *4* | *9* | 37 |
| SER | + | 0 | 0 | *12*[c] | *14*[c] | *1*[c] | 27 |
| | - | 0 | 2 | *1* | *4* | *8* | 15 |

[a] See Table 4 for abbreviations of aminopeptidase reactions.

[b] Identified by the Rapid ID32A kit.

[c] $\chi^2$ analysis; all $P < 0.001$.

$H_o$: There is no association between gelatin hydrolysis and the distribution of certain positive aminopeptidase reactions for the 5 *Peptostreptococcus* species.

# APPENDIX D.

## CURRICULUM VITAE.

JAMES NG

### EDUCATION.

1976-1982 - Jarvis Collegiate Institute
Toronto, Ontario
S.S.H.G.D. (Grade 13)
Ontario Scholar

1982-1987 - University of Toronto
Toronto, Ontario
Specialist in Microbiology
Bachelor of Science degree

1988-1991 - Carleton University
Ottawa, Ontario
Master of Science
Department of Biology

1991- 1996 - University of Ottawa
Ottawa, Canada
Doctor of Philosophy
Department of Biology

### THESIS.

**Ng, J.** 1991. Recombination and transposition events mediated by the insertion sequence IS*1071*. M. Sc. thesis. Institute of Biology, Carleton University, Ottawa, Ontario, Canada.

### PEER REVIEWED PUBLICATIONS.

**Nakatsu, C., J. Ng, R. Singh, N. Straus, and C. Wyndham.** 1991. Chlorobenzoate catabolic transposon Tn*5271* is a composite class I element with flanking class II insertion sequences. Proc. Natl. Acad. Sci. USA 88:8312-8316.

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**Wyndham, R. C., C. Nakatsu, M. Peel, A. Cashore, J. Ng, and F. Szilagy.** 1994. Distribution of the catabolic transposon Tn*5271* in a groundwater bioremediation system. Appl. Environ. Microbiol. **60:**86-93.

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**Ng, J., L.-K. Ng, E. Holst, and J. R. Dillon.** 1996. Differentiation of protein L-containing and albumin-binding *Peptostreptococcus magnus* isolates by DNA amplification, ribotyping, and pulsed field gel electrophoresis. Anaerobe **2:**95-102.

**MANUSCRIPTS IN PREPARATION.**

**Ng, J., L.-K. Ng, D. Mayrand, and J. R. Dillon.** 1996. The proteolytic activity of five *Peptostreptococcus* species on gelatin is correlated with aminopeptidase activity. In preparation.

**Ng, J., L.-K. Ng, and J. R. Dillon.** 1996. *Peptostreptococcus* species are important pathogens in the female genital tract. In preparation.

**PUBLISHED ABSTRACTS.**

**Ng, J., and R. C. Wyndham.** 1990.Unusual genetic rearrangements associated with Tn*5* mutagenesis in *Alcaligenes* sp. BR60. The Canadian Society for Microbiologist meeting. Calgary, Alberta.

**Nakatsu, C., J. Ng, D. Wiens, and R. C. Wyndham.** 1991. Catabolic transposon Tn*5271*: Structure, transposition and organization of chlorobenzoate catabolic genes. The American Society for Microbiologist meeting. Dallas, Texas, USA.

**CONFERENCE PRESENTATIONS.**

**Ng, J., D. Wiens, and R. C. Wyndham.** 1991. Recombination and transpositional events mediated by the insertion sequence IS*1071*. The Canadian Society for Microbiologist meeting. London, Ontario. Oral presentation.

**Ng, J., L.-K. Ng, and J. R. Dillon.** 1993. Analysis of proteolytic activity observed by *Peptostreptococcus magnus* isolates from female genital tract infections. The Canadian Bacterial Diseases Network. Vancouver, B. C. Poster presentation.

**Ng, J., L.-K. Ng, and J. R. Dillon.** 1994. Clonal analysis of protein L positive and negative *Peptostreptococcus magnus* isolates: PCR-RFLP, ribotyping, and pulsed-field gel electrophoresis. The Canadian Society for Microbiologists/ Canadian Bacterial Diseases Network meeting. Vancouver, B. C. Oral presentation.