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Enzymatically Active *Peptostreptococcus magnus*: Association with Site of Infection

CANDACE J. KREPEL,[1,2]* CLAUDIA M. GOHR,[1,2] ALONZO P. WALKER,[2] SILAS G. FARMER,[3] AND CHARLES E. EDMISTON[1,2,3]

Surgical Microbiology Research Laboratory[1] and Departments of Surgery[2] and Pathology,[3] Medical College of Wisconsin, Milwaukee, Wisconsin 53226

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Fifty-four strains of *Peptostreptococcus magnus* (11 were recovered from abdominal infections, 18 were from nonpuerperal breast abscesses, and 21 were from diabetic foot infections; the type strain and three other strains were from the American Type Culture Collection, Rockville, Md.) and the type strain of *Peptostreptococcus micros* were tested for their ability to produce various enzymes, including catalase, hippurate hydrolase, serine dehydratase, threonine dehydratase, collagenase, gelatinase, alkaline phosphatase, and esterase C4. The data were analyzed by cluster analysis. The results showed that all but one strain could be assigned to either of two distinct, valid clusters. The first cluster of 11 strains was composed of strains that were relatively inactive, having produced one or two of the eight strain-dependent enzymes. The second was a large cluster of strains ($n = 43$) that were considerably more active, all having produced at least three enzymes; the vast majority of strains (89%) produced four or more enzymes. The unclustered strain produced one enzyme that was different from that produced by the strains in the first cluster. The χ^2 test of homogeneity applied to the clustering solution indicated that greater enzyme activity was significantly associated with the site of infection ($P < 0.001$). The more enzymatically active *P. magnus* strains were recovered significantly more often from nonpuerperal breast abscesses and diabetic foot infections than they were from abdominal infections. These results may provide insight into the nature of certain polymicrobial soft tissue infections and suggest that (i) *P. magnus* may participate more in nonpuerperal breast and diabetic foot infections than in abdominal infections and that (ii) peptostreptococcal production of proteolytic enzymes may have an important adjunctive effect on the pathogenesis of certain soft tissue infections.

Peptostreptococcus magnus is a common clinical isolate that is frequently recovered from infections of soft tissues and the peritoneal cavity. Although its role in infection is unclear, the frequent recovery of *P. magnus* from polymicrobial infections suggests that these organisms contribute to the infection process. Recovery of *P. magnus* from intra-abdominal infections is well documented (1, 17).

Edmiston et al. (5) reported that two-thirds of all organisms recovered from 56 women with nonpuerperal breast abscesses were anaerobes; *P. magnus* was the most commonly isolated anaerobic species. Habif et al. (7) have suggested that breast abscesses arise as a result of infection in a major lactiferous duct which is lined with squamous epithelial cells and which is subsequently blocked by keratin plugs. Leach et al. (13) have reported a relationship between recent vaginal manipulation and the development of breast abscesses. The roles of the various organisms in the process are not known.

Diabetic patients are prone to the development of soft tissue infections of the foot because of ischemia and neuropathy. *P. magnus* has been reported to be frequently isolated from these types of infections (14, 16, 18, 21). These findings suggest that *P. magnus* is opportunistic, and under favorable conditions it can be a pathogen with significant potential for morbidity and mortality.

A polymicrobial facultatively anaerobic flora can be recovered from most anaerobic infections. The presence of multiple organisms permits synergistic interactions, which augment the virulence of the individual organisms, allowing them to profit from metabolic and enzymatic associations. The opportunistic nature of *P. magnus* and its interrelationship with other bacterial flora and with the human host is poorly understood. In the investigation described here, we attempted to elucidate the role of *P. magnus* in three specific polymicrobial environments: intra-abdominal infections, nonpuerperal breast abscesses, and diabetic foot ulcers.

MATERIALS AND METHODS

During an 8-year period, 222 clinical specimens from intra-abdominal infections, 58 specimens from nonpuerperal breast abscesses, and 56 from diabetic foot ulcers were cultured. All specimens were collected anaerobically and were transported within 2 h of collection to the Surgical Microbiology Research Laboratory. They were inoculated onto prereduced Center for Disease Control formulation anaerobic agar plates with sheep erythrocytes; laked sheep blood, kanamycin, and vancomycin; and sheep erythrocytes and phenylethyl alcohol (Remel, Lenexa, Kans.) in a Coy anaerobic chamber. Tissue specimens were ground in 1 ml of Wilkins-Chalgren broth with a conical tissue homogenizer (Bellco Glass, Vineland, N.J.). Swab specimens were vortexed in 1 ml of reduced brain heart infusion broth for 15 s, and the broth was inoculated onto plates. Media were incubated at 35°C for 48 h in an anaerobic chamber prior to colony type isolations.

Fifty strains of obligately anaerobic gram-positive cocci were recovered and identified as *P. magnus* by the methods of Holdeman et al. (8). Strains that showed weak alkaline phosphatase activity (API ZYM; Analytab Products, Plainview, N.Y.), but no gelatinase or catalase activity, were

* Corresponding author.

distinguished from *Peptostreptococcus micros* by demonstration of a uniform cell size of ≥0.8 μm in diameter (6). Strains were stored at −70°C in double-strength skim milk.

A total of 54 strains of *P. magnus* were studied; 11 strains were from intra-abdominal infections, 21 were from nonpuerperal breast abscesses, and 18 were from diabetic foot ulcers; four reference strains of *P. magnus* (ATCC 15794^T, ATCC 14955^T, ATCC 14956^T, ATCC 29328^T) were obtained from the American Type Culture Collection (ATCC; Rockville, Md.). ATCC 33270, the type strain of *P. micros*, a species closely related to *P. magnus*, was also obtained from ATCC. The study strains were randomly assigned numbers from 1 to 55 and were stored (−70°C) until further testing. Because all strains were identified by number only, the investigators were blinded to the strain source site until the end of the study.

All strains were tested for phenotypic characteristics which have been reported to be strain dependent. Gelatin liquefaction was tested by inoculating each strain into a tube of prereduced, anaerobically sterilized peptone yeast broth with 0.5% gelatin. Tubes were incubated at 35°C for 4 weeks and were read as described previously (8). Production of ammonia from serine and threonine by dehydratase enzymes was assessed as described previously (2) by using freshly prepared peptone yeast extract broth with a 0.7% concentration of filter-sterilized aqueous amino acid. Catalase production was assessed as described previously (8). The ability of strains to hydrolyze hippurate was tested by using hippurate disks (21-085; Remel), according to the manufacturer's instructions. Assays for alkaline phosphatase and esterase C4 activities were performed with the API ZYM system according to the manufacturer's instructions. Collagenase activity was assayed by the method of Steffen and Hentges (20).

The χ^2 test of homogeneity was applied to the results of each of the eight phenotypic characteristics. Because the expected counts were less than five in more than 20% of the cells for five of the characteristics, the results of three characteristics are reported (3).

Cluster analysis was performed on the complete data set with the SPSS-X (SPSS, Inc., Chicago, Ill.) statistical data analysis program by using the group average method. The phenotypic characteristics were coded in a simple binary fashion: a value of 1 was assigned if a strain was positive for a characteristic, or a value of 0 was assigned if it was negative for a characteristic. The analysis was done three times by using the following different measures of distance: the binary squared Euclidean distance measure, the phi similarity index, and the Dice similarity index. Since the clustering algorithm operates on distances, the similarity matrices were reversed to transform the values to dissimilarities.

The stopping rule of Krzanowski and Lai (12) was used to determine which step of the cluster analysis solution was the most appropriate stopping point. A modification of Milligan's (15) internal criterion was used to assess the internal cohesion of the clusters at the stopping point. The 55 strains were randomly assigned to the number of clusters present at the stopping point by using the Lotus 1-2-3 (Lotus Development Corporation, Cambridge, Mass.) random number-generating function. This was done a total of 20 times. The means and standard deviations of the between-cluster variations were computed, and they served as the comparators for the significance of the internal criterion computed from the solution.

The χ^2 test of homogeneity was applied to the results of the cluster solution to discern any association between the site where a strain was recovered and the cluster to which it was assigned.

TABLE 1. Number of strains producing enzymes, by site of recovery

Enzyme	No. of strains[a]				
	A (n = 11)	Br (n = 21)	D (n = 18)	Mg (n = 4)	Mc (n = 1)
Collagenase ($P < 0.001$)[b]	2	17	17	4	
Serine dehydratase	5	18	18	4	
Catalase		2	7		
Gelatinase ($P < 0.02$)[b]	1	13	8	1	
Hippurate hydrolase ($P < 0.05$)[b]	2	12	12	3	
Threonine dehydratase	2	5	2	1	
Alkaline phosphatase	10	20	16	4	1
Esterase C4	1	1			1

[a] A, abdominal infection; Br, nonpuerperal breast abscess; D, diabetic foot ulcer; Mg, ATCC strains *P. magnus*; Mc, ATCC strain *P. micros*.
[b] Significantly associated with site of infection.

RESULTS

P. magnus was recovered significantly more often from nonpuerperal breast abscesses and diabetic foot ulcers than it was from intra-abdominal infections, as reported previously (11). All but one of the strains were recovered in mixed culture; 48 of 49 cultures yielded both facultative and obligate anaerobes. The phenotypic characteristics of the strains are listed in Table 1. Collagenase production was associated with the site of infection ($P < 0.001$; χ^2 test of homogeneity), as was production of gelatinase ($P < 0.02$) and hippurate hydrolase ($P < 0.05$).

Constitutive enzyme profiles, as assayed by the API ZYM system, were remarkably consistent. No strain, including the type strain of *P. micros*, produced valine or lysine aminopeptidase, trypsin, chymotrypsin, α- or β-galactosidase, β-glucuronidase, α- or β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, or lipase C14. All strains produced esterase lipase C6, leucine aminopeptidase, and phosphohydrolase. Only a single strain (A349-9) was negative for acid phosphatase. All but three strains (*P. magnus* A284-5 and Br37-2 and *P. micros* ATCC 33270^T) were negative for esterase C4 production, and only four strains (*P. magnus* A349-9, Br58-9, D155-s5, and D156-17) failed to produce detectible amounts of alkaline phosphatase.

The SPSS-X cluster analysis program output included the matrix of distances between strains, a schedule of the steps in the cluster solution, including the coefficient of distance between each step and the next one, a horizontal icicle plot (data not shown), and a dendrogram constructed from the matrix of distances.

The clustering algorithm for the dendrogram (Fig. 1) operates from left to right, with strains being joined at each step. Thus, at the beginning, there were 55 strains and 55 clusters, and at the end, all cases were in a single cluster. The distances between clusters were rescaled such that all fell between 1 and 25.

The stopping rule of Krzanowski and Lai (12) was applied, and the plot of criterion ratio suggested 4, 11, and 16 clusters as possible stopping points (data not shown). By using the modification of Milligan's (15) internal criterion, the mean of

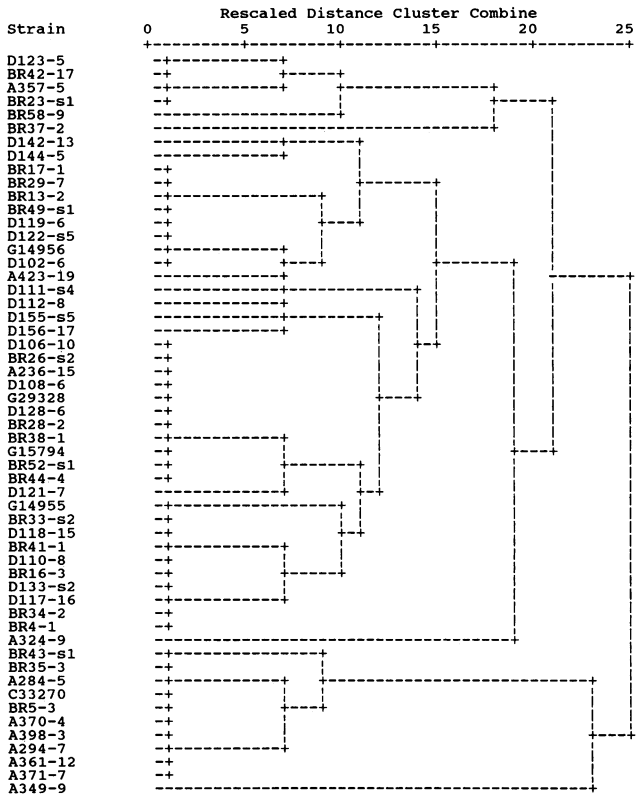

FIG. 1. Dendrogram made by using average linkage between groups.

the between-cluster variance (internal criterion) of random clusterings of the strains at four clusters was calculated to be 2,840, with a standard deviation of 32.56. The internal criterion for the phenotypic data at four clusters was 3,133. This value was 9 standard deviations beyond the mean for randomly clustered strains, indicating that the cluster solution had considerable internal cohesion.

The cluster solutions for the phenotypic characteristics by using either reversed phi or reversed Dice measures were very similar to the solution obtained by using binary squared Euclidean distance (data not shown).

DISCUSSION

Cluster analysis is a technique for identifying relatively homogeneous subsets within the total group of cases on the basis of individual case values for chosen variables. A number of statistical techniques are available for comparing any number of groups on the basis of a single variable. Cluster analysis is unique in its ability to consider multiple variables simultaneously. However, care must be taken to select relevant variables, because the results of the analysis are dependent on the variables chosen. The variables selected for this analysis included all phenotypic characteristics known to be strain dependent, that is, all known variables that could differentiate between two strains of *P. magnus*. The solution must satisfy the requirements for external isolation and internal cohesion (15). External isolation is defined as the isolation of cases in one cluster from cases in another cluster by relatively open areas of hyperspace. Internal cohesion requires that the cases in a cluster be similar, but probably not identical, to each other (4).

TABLE 2. Cluster assignment determined by using three measures of distance

Cluster	Strain by the following measure of distance[a]:		
	BSED	Phi	Dice
Unclustered	A349-9	A349-9	A349-9 Br37-2
1	*P. micros* Br5-3 Br35-3 Br43-s1 A284-5 A294-7 A361-12 A370-4 A371-7 A398-3	*P. micros* Br5-3 Br35-3 Br43-s1 A284-5 A294-7 A361-12 A370-4 A371-7 A398-3 A423-19	*P. micros* Br5-3 Br35-3 Br43-s1 A284-5 A294-7 A361-12 A370-4 A371-7 A398-3 A423-19
2	A357-5 Br23-s1 Br37-2 Br42-17 Br58-9 D123-5	A357-5 Br23-s1 Br37-2 Br42-17 Br58-9 D123-5	
3	Balance of strains	Balance of strains	Balance of strains

[a] BSED, Binary squared Euclidian distance; Phi, reversed phi similarity index; Dice, reversed Dice similarity index.

Thus, two cases with very similar values for all variables are placed in the same cluster, while a case with quite different values for one or more variables is placed in a different cluster.

Agglomerative clustering algorithms begin with one cluster of two cases and end with all cases in one cluster. The stopping rule of Krzanowski and Lai (12) was used in the present study to determine an appropriate stopping point. This satisfied the requirement for external isolation. Any clustering solution must be evaluated for internal cohesion to distinguish real from arbitrary clustering. A modification of the internal criterion of Milligan (15) was used. Larger values of the internal criterion, which is the sum of all between-cluster variances, reflect a better fit between the data and the clusters. If the between-cluster variance of the solution exceeds the mean of the between-cluster variance of random clusters by more than 2 standard deviations, the null hypothesis that the solution is merely a random clustering (there is no true structure) may be rejected.

Given that there were 55 strains in the problem set, the four-cluster solution was chosen as the stopping point since use of a cluster solution at a higher number of clusters would have created artificial subclusters as well as a number of unclustered, individual cases. It was obvious that a cluster structure existed since the internal criterion for the four-cluster solution was 9 standard deviations above the mean for cases randomly distributed to four clusters. When the cluster solutions obtained by using the three measures were compared, they were remarkably similar. Table 2 shows the pertinent features of the three solutions. Strain A423-19 was assigned to cluster 1 by two of the three solutions. An examination of the strain's characteristics showed that it produced two of the eight enzymes included in the analysis. The other strains assigned to cluster 1 also produced a

maximum of two of the eight enzymes. Therefore, A423-19 was assigned to cluster 1.

When the phenotypic characteristics of the strains in each cluster were examined, it was found that the first cluster of 11 strains was composed of strains that were relatively inactive, all having produced alkaline phosphatase and 5 having produced one of the other enzymes examined in this study. The second cluster of six strains contained strains that were considerably more active, having produced four to six enzymes each. The last cluster (37 strains) contained strains which produced at least three enzymes; the vast majority (89%) produced four to six enzymes.

The strains in clusters 2 and 3 were combined in the same cluster in the four-cluster solution by using the reversed Dice measure. When the dendrogram for this solution (data not shown) was examined, the distance between the four-cluster step and the last step was over half of the total distance, which indicated that four clusters was an appropriate stopping point. The characteristics of the strains in the binary squared Eucliden distance and phi similarity index cluster 2 differed from those in cluster 3 only by which of the four to six enzymes that the strains produced. The next step in both of these solutions combined cluster 2 with cluster 3. The rescaled distance at this step was relatively small. Therefore, it seemed reasonable to consider that cluster 1 contained 11 strains (*P. micros* and *P. magnus* Br5-3, Br35-3, Br43-s1, A284-5, A294-7, A361-12, A370-4, A371-7, A398-3, and A423-19) and that cluster 2 contained the balance of the strains except for strain A349-9, which stood alone.

The solution presented above appears to be contradictory in the case of the type strain of *P. micros*, which was included in a cluster of *P. magnus* strains. However, it is entirely possible that the *P. micros* strain was not correctly identified. The majority of type strains for bacterial species recognized in the *Approved Lists of Bacterial Names* (19) were described prior to 1979. This was before the widespread availability of technologies such as DNA hybridization and homology, nucleotide sequencing of the 16S subunit of rRNA, and whole-cell fatty acid analysis. The species were characterized solely on the basis of their phenotypic characteristics. To date, little work has been done on the genus *Peptostreptococcus*. Cato et al. (2) suggested that two strains identified as *P. magnus*, type strain ATCC 15794 and ATCC 14955, originally deposited as the type strain of "*Micrococcus variabilis*," are distinct, although they refrained from proposing a revival of the *M. variabilis* name.

Because *P. micros* and *P. magnus* are so difficult to distinguish, it is also possible that the other strains assigned to cluster 1 may, in fact, be strains of *P. micros*. Strain A349-9 may or may not be one of the two species in question. It is closely related, because the original identifying characteristics classified it as a *P. magnus* strain. The benefit of using cluster analysis as described here is that the separation between strains is based not on traditional biochemical characteristics but, rather, on the production of enzymes that may have an impact on the disease process in the host.

The χ^2 test of homogeneity was applied to the clustering solution, and the results indicated that greater enzyme activity is significantly associated with the site of infection ($P < 0.001$). That is, there was a much higher probability that strains recovered from nonpuerperal breast abscesses or diabetic foot ulcers were assigned to the more active cluster rather than the relatively inactive cluster. This is consistent with the results of the association of positivity of individual tests with the site of infection.

The association of collagenase-producing strains with the site of infection was particularly interesting. Previous work with collagenase-producing strains of *Porphyromonas gingivalis*, a species found to be associated with periodontal disease, has shown that strains demonstrating high levels of enzyme activity produce experimental mixed infections more rapidly than do strains with little or no enzyme activity (9). Kestenbaum et al. (10) have shown that the ability of oral fusiforms to induce skin lesions in rabbits is enhanced by cell extracts of collagenolytic *P. gingivalis*. A similar process of enhancement may take place in soft tissue infections involving collagenase-producing *P. magnus* strains. The cluster analysis described here demonstrated that it can be used to identify groups of strains by functional characteristics and offers the opportunity of relating the characteristics of those groups to specific disease processes. All of the strains in the less enzymatically active group produced alkaline phosphatase, two strains produced hippurate hydrolase, two strains produced esterase C4, and one strain produced serine dehydratase. Most of these strains came from acute abdominal infections dominated by *Bacteroides fragilis*, *Escherichia coli*, or both. It is unlikely that *P. magnus* played a large role in these cases, especially considering the low enzymatic activity of *P. magnus*. In contrast, the strains clustered into the more enzymatically active group, which included all strains that produced the proteolytic enzymes collagenase and gelatinase, were recovered predominantly from soft tissue infections, which tend to be slow, insidious disease processes. The nonpuerperal breast abscesses were primarily nonacute, characterized by chronic infection and inflammation and subareolar mass, with or without a sinus tract and suppuration (5). The diabetic foot infections in the current study were all progressive and necrotizing infections requiring surgical debridement or amputation. The finding that the proteolytic strains of *P. magnus* are associated with infections causing tissue destruction may provide new insight into these disease mechanisms.

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