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Evolution of Alkaline Phosphatase in Marine Species of *Vibrio*

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The evolution of alkaline phosphatase was studied in marine species of *Vibrio*. Two antisera prepared against purified alkaline phosphatases from *Vibrio splendidus* and *Vibrio harveyi* were used to estimate the amino acid sequence divergence of this enzyme in 51 strains belonging to nine species. The methods used were the quantitative microcomplement fixation technique and the Ouchterlony double-diffusion procedure. There was a high degree of congruence between the measurement of the amino acid sequence divergence of alkaline phosphatase and the percentage of deoxyribonucleic acid homology of the different organisms relative to both reference strains (correlation coefficient of −0.89) as well as between the amino acid sequence divergence of alkaline phosphatase and superoxide dismutase (correlation coefficient of 0.92) relative to *V. splendidus*. These findings supported the view that the evolution of marine species of *Vibrio* is primarily vertical and that horizontal evolution (involving genetic exchange between species), if significant, is restricted to a minor fraction of the bacterial genome.

There are currently two distinct and opposing views on bacterial evolution and speciation (18, 31, 36). One view holds that most of the bacterial genome evolves vertically in that it is genetically isolated from other bacterial species. The second view states that horizontal evolution (the genetic exchange between species of the same and different genera) occurs at a high frequency, and, therefore, all bacteria share a common gene pool. The latter view is based primarily on the ubiquity of genetic exchange mechanisms in bacteria and particularly on the potential of plasmid transfer between distantly related organisms. These findings are documented by a large body of literature (7, 9, 31) which tends to overwhelm the relatively fewer studies suggesting that genetic exchange between species may not be an important factor in their evolution (7). The consequences of the presence or absence of genetic exchange are profoundly different and experimentally determinable (31, 36). Extensive horizontal evolution would obscure sharp phenotypic and genotypic differences between organisms and would make bacterial species identification an unattainable goal, whereas vertical evolution would result in species recognizable on the basis of phenotypic and genotypic discontinuities. The latter view is supported by many thorough taxonomic studies which indicate the existence of such discontinuities among procaryotes (5, 31). Evidence for vertical or horizontal evolution may be obtained by a comparison of the sequence divergence of different informational molecules (31, 36). Congruence between their sequence changes relative to a single reference organism would be consistent with vertical evolution, whereas a lack of congruence would be consistent with horizontal evolution.

Previously, high degrees of congruence (correlation coefficients of over 0.9) have been observed between the sequence changes of rRNA, glutamine synthetase (EC 6.3.1.2), and the iron-containing superoxide dismutase (EC 1.15.1.1) from species of *Vibrio, Photobacterium,* and *Aeromonas,* as well as a number of species from different genera of the *Enterobacteriaceae* (2). These informational molecules have sequences which are highly conserved, and all are indispensable since they perform essential metabolic functions. In addition, rRNA and glutamine synthetase are parts of different, complex, functionally interdependent units which must have evolved in concert and are probably poised at their optimal functional state (20). Consequently, it seems unlikely that selective conditions favoring the exchange of genes coding for these properties would occur in nature.

Some of these strictures may not apply to alkaline phosphatase (EC 3.1.3.1), a dispensable, periplasmic protein which does not appear to be part of a complex, interdependent functional unit (21, 30). Relative to glutamine synthetase, the amino acid sequence of this enzyme is not highly conserved (4, 12). Since alkaline phosphatase is not always present in different strains of a species, and since selective pressure favoring

the acquisition of this enzyme could be expected to occur in nature, it is possible that alkaline phosphatase could be restored by genetic exchange. If the genetic donor were from a different species, congruence between alkaline phosphatase and other informational molecules would not be observed.

The present work involves a determination of the amino acid sequence divergence of the alkaline phosphatases from marine species of *Vibrio* and a comparison of the results with previous studies of informational molecules within this genus. The methods used were Ouchterlony double diffusion and the quantitative microcomplement fixation technique that was extensively applied to studies of protein evolution by Wilson and his collaborators (10). The latter provides an estimate of the amino acid sequence difference between the reference alkaline phosphatase and the enzyme from another strain. This divergence is expressed as immunological distance (ImD), a parameter proportional to percentage of amino acid sequence difference (11, 19).

MATERIALS AND METHODS

Bacterial strains. All of the strains used in the microcomplement fixation experiments have been previously characterized in our laboratory (5, 29) and are listed in Table 3. Many of the strains considered were previously placed into *Beneckea* and *Lucibacterium* but are currently assigned to *Vibrio* (6), a treatment which will be followed in the ninth edition of *Bergey's Manual of Determinative Bacteriology*. Roman numerals following species names are used to designate biotypes. Single strains or small groups of strains not belonging to any of the described species are unnamed pending the characterization of additional isolates (5). All species, biotypes, or groups contain strains related by over 80% DNA homology (29).

Enzyme assay. Alkaline phosphatase activity was measured spectrophotometrically by following the increase in absorbance at 410 nm due to the release of *p*-nitrophenol from *p*-nitrophenyl phosphate. The assay mixture (1.0 ml) contained 0.6 M Tris-hydrochloride (pH 8.2), 1 mM $MgCl_2$, and 1 mM *p*-nitrophenyl phosphate (34); the reaction was started by the addition of substrate. One unit of activity was defined as the amount of enzyme catalyzing the release of 1 μmol of *p*-nitrophenol per min at 25°C. Specific activity is expressed as units per milligram of protein; protein was determined by the method of Lowry et al. (25) with bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis under nondenaturing conditions was performed as described by Davis (13), and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as detailed by Laemmli (22). Gels were stained for protein with Coomassie blue (14), and alkaline phosphatase activity was localized by the method of Gomori (16).

Purification of alkaline phosphatases. Cultures were incubated on a rotary shaker at 28°C and harvested in early stationary phase; growth was limited by P_i depletion to derepress alkaline phosphatase (33). Unless otherwise stated, all steps involved in the enzyme purifications were performed at 4°C. Pooled enzyme fractions were concentrated by ultrafiltration using an Amicon stirred cell with a PM10 filter (Amicon Corp., Lexington, Mass.).

Vibrio splendidus II strain 2 was grown in a marine basal medium (27) containing 0.10 mM K_2HPO_4 and supplemented with 30 mM glycerol and 0.05% (wt/vol) Casamino Acids. Cells were osmotically shocked as described by Hayashi et al. (17), and the shock fluids were stored at −15°C. The combined osmotic shock fluids were fractionated with solid $(NH_4)_2SO_4$, and the protein which precipitated between 60 and 80% saturation was suspended in 100 mM Tris-hydrochloride (pH 7.5) containing 50 mM $(NH_4)_2SO_4$ (necessary for the stabilization of alkaline phosphatase activity in this strain), 10 mM $MgCl_2$, and 0.1 mM $ZnSO_4$ (TAMZ). After extensive dialysis against TAMZ, the protein was applied to a DEAE-Sephadex column (Pharmacia Inc., Uppsala, Sweden) equilibrated with the same buffer, and the alkaline phosphatase was eluted with a linear gradient of 0 to 500 mM NaCl in TAMZ (activity peak at 90 mM NaCl). Fractions containing over 10% of the peak activity were pooled, concentrated by ultrafiltration, and applied to a Sephadex G-100 column (Pharmacia Inc.) equilibrated with TAMZ. Alkaline phosphatase activity eluted just after the void volume. Fractions containing over 35% of the peak activity were pooled, concentrated by ultrafiltration, dialyzed against 100 mM Tris-hydrochloride (pH 8.4) containing 50 mM $(NH_4)_2SO_4$, and subsequently applied to an affinity column (type 1). The affinity column (8) was prepared by diazotization of 4-(*p*-aminophenyl-azo)phenylarsonic acid to tyraminyl-Sepharose (Sepharose from Pharmacia Inc.). The arsonate moiety is an analog of phosphate and served as the biospecific ligand. Alkaline phosphatase was eluted with a linear gradient of 0 to 80 mM K_2HPO_4 in 50 mM $(NH_4)_2SO_4$ and 100 mM Tris-hydrochloride (pH 8.4). The activity peak eluted at 18 mM K_2HPO_4. Fractions having over 15% of the peak activity were combined, concentrated by ultrafiltration, dialyzed against 100 mM Tris-hydrochloride (pH 7.5) containing 50 mM $(NH_4)_2SO_4$, and stored at −15°C.

Vibrio harveyi strain 392 was grown in a terrestrial basal medium (28) containing 0.10 mM K_2HPO_4 and supplemented with 250 mM NaCl, 20 mM glycerol, and 0.1% (wt/vol) yeast extract. After harvesting, the cells were washed in a solution composed of 100 mM Tris-hydrochloride (pH 7.5), 250 mM NaCl, 10 mM KCl, 2 mM $MgSO_4$, and 0.55 mM $CaCl_2$ and then stored at −15°C. A total of 890 g (wet weight) of cells was suspended in 50 mM Tris-hydrochloride (pH 7.5) containing 10 mM $MgCl_2$. The cells were disrupted by ultrasonic treatment, and nucleic acids were removed by streptomycin sulfate precipitation as previously described (3). Samples of the cell-free extract (50 ml) were heated for 3 min at 65°C and centrifuged, and the protein in the supernatant was fractionated with solid $(NH_4)_2SO_4$. The protein precipitating between 60 and 80% saturation was redissolved in 10 mM Tris-hydrochloride (pH 7.5) containing 50 mM NaCl and 1 mM $MgCl_2$, dialyzed extensively against this buffer,

and applied to a DEAE-Sephadex column equilibrated with the same buffer. A linear gradient of 50 to 750 mM NaCl in 10 mM Tris-hydrochloride (pH 7.5) and 1 mM $MgCl_2$ was used to elute the enzyme (activity peak at 300 mM NaCl). Fractions containing over 5% of the peak activity were pooled, dialyzed against 10 mM Tris-hydrochloride (pH 8.2) containing 1 mM $MgCl_2$ (TM), and placed on a DEAE-cellulose column (Reeve-Angel, Clifton, N.J.) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of 0 to 700 mM NaCl in TM; the activity peak was found at 125 mM NaCl. Fractions containing over 33% of the peak activity were pooled, concentrated by ultrafiltration, dialyzed against TM, and applied to a Sephadex G-100 column equilibrated with TM. Alkaline phosphatase eluted just after the void volume. The purity of the fractions containing alkaline phosphatase activity was determined by electrophoresis in 7.5% acrylamide gels performed under nondenaturing conditions. Fractions containing only a single protein band were combined, dialyzed against 10 mM Tris-hydrochloride (pH 8.2), and applied to an affinity column (type 2) equilibrated with the same buffer. The affinity column was L-histidyl-Sepharose coupled by diazotization to 4-aminobenzylphosphonic acid (23). The enzyme was eluted by a linear gradient of 0 to 80 mM K_2HPO_4 in 10 mM Tris-hydrochloride (pH 8.2), with the peak activity found at 3 mM K_2HPO_4. Fractions containing over 19% of the peak activity were pooled, concentrated by ultrafiltration, dialyzed against 25 mM Tris-hydrochloride (pH 7.5), and stored at −15°C.

Preparation of antisera. Purified alkaline phosphatases from *V. splendidus* II strain 2 and *V. harveyi* strain 392 were injected into four and three male New Zealand white rabbits, respectively, using the procedure and schedule of Champion et al. (11). Each rabbit received a total of 3.5 to 6.0 mg of the antigen. Rabbits were bled, and the antisera were treated as previously described (10). The antisera were titrated against the homologous antigen and pooled in inverse proportion to their titers. Only pooled antisera were used in the experiments.

Preparation of cell-free extracts for immunological experiments. Alkaline pbosphatase was derepressed by growing strains in media containing 0.1 mM K_2HPO_4, 30 mM glycerol, and 0.1 to 0.2% (wt/vol) yeast extract. Terrestrial basal medium (28) supplemented with 50 mM NaCl was used for the cultivation of *Vibrio cholerae, Vibrio metschnikovii,* and *Escherichia coli.* All of the remaining strains were grown in a marine medium (27). The cultures were incubated at 24 or 30°C on a rotary shaker and harvested in early stationary phase. Alkaline phosphatase from all strains of *V. splendidus* and from strain 16 was released by an osmotic shock procedure (17). Cells of all other strains were disrupted by ultrasonic treatment, and the nucleic acids were removed by streptomycin sulfate precipitation (3). Extracts prepared by both methods were fractionated with $(NH_4)_2SO_4$. The protein precipitating between 55 and 85% saturation was redissolved in 50 mM Tris-hydrochloride (pH 7.5) containing 10 mM $MgCl_2$, dialyzed extensively against this buffer, and stored at −15°C.

Immunochemical procedures. Immunodiffusion was performed as described by Munoz (26) using 1% Ionagar (Wilson Diagnostics, Glenwood, Ill.) with 10 mM Tris-hydrochloride (pH 7.5), 150 mM NaCl, and 1 mM $MgCl_2$. Cell-free extracts from at least two strains of each species, biotype, or group were used in the immunodiffusion experiments. In the case of *V. splendidus,* all strains containing detectable alkaline phosphatase (see Table 3) were tested. The immunodiffusion plates were incubated at 16°C and examined several times between 24 and 48 h.

Immunoelectrophoresis was performed according to the method of Williams (35), using 1% Ionagar with 2.1 mM KH_2PO_4 and 22.9 mM K_2HPO_4 (pH 7.8).

The quantitative microcomplement fixation method used in these studies has been described by Champion et al. (10). A typical experiment consisted of five concentrations of the heterologous antigen (in a twofold dilution series) reacted against four different antiserum dilutions selected to fix 30 to 85% of the complement at the equivalence point. The same experiment included five concentrations of the homologous antigen tested against a dilution of antiserum which resulted in 75% of the complement being fixed at the equivalence point. A control without antiserum was included for each antigen concentration, and a control, in duplicate, was included for each antiserum dilution. The titer was designated as the antiserum dilution at which 75% of the complement was fixed at the equivalence point. ImD was calculated from experimentally determined titers using the equation of Champion et al. (10): ImD = 100 log (heterologous titer/homologous titer).

RESULTS

Purification of alkaline phosphatases. Summaries of the purifications of alkaline phosphatase from *V. splendidus* II strain 2 and *V. harveyi* strain 392 are presented in Table 1 and 2, respectively. In every step involving column chromatography, the alkaline phosphate activity eluted as a single, nearly symmetrical peak. The type 1 affinity column (see Materials and Methods) used to purify the enzyme from *V. splendidus* continuously leached a chromophoric substance (even after extensive rinsing) which precluded the measurement of protein at 280 nm. This problem was not encountered with

TABLE 1. *Purification of alkaline phosphatase from V. splendidus II strain 2*

Purification step	Total activity (units)[a]	Sp act[b]	Purification factor	Recovery (%)
Osmotic shock	38,470	9.8	1	100
60 to 80% $(NH_4)_2SO_4$	28,067	20	2	73
DEAE-Sephadex	22,539	425	43	59
Sephadex G-100	16,809	763	78	44
Type 1 affinity column	9,545	1,061	108	25

[a] Activity catalyzing the release of 1 μmol of *p*-nitrophenol per min at 25°C.

[b] Units per milligram of protein.

TABLE 2. *Purification of alkaline phosphatase from V. harveyi strain 392*

Purification step	Total activity (units)[a]	Sp act[b]	Purification factor	Recovery (%)
Cell-free extract	435,802	5.2	1	100
Streptomycin sulfate	414,546	9.6	1.8	95
Heat treatment (65°C)	382,250	36	7	88
60 to 80% $(NH_4)_2SO_4$	333,951	153	29	77
DEAE-Sephadex	286,063	326	63	66
DEAE-cellulose	221,314	469	90	51
Sephadex G-100	93,999	740	142	22
Type 2 affinity column	83,352	896	173	19

[a] Activity catalyzing the release of 1 μmol of *p*-nitrophenol per min at 25°C.
[b] Units per milligram of protein.

the type 2 affinity column used in the purification of alkaline phosphatase from *V. harveyi*.

Criteria for alkaline phosphatase and antiserum purity. Alkaline phosphatases from both reference strains migrated as single protein bands when 15-μg quantities were electrophoresed in 6, 7.5, and 9% (wt/vol) acrylamide gels under nondenaturing conditions. With both alkaline phosphatases, the relative mobility of the enzyme localized by the activity stain was identical to that of the protein band. A further indication of enzyme purity was the presence of one protein band in sodium dodecyl sulfate-polyacrylamide (8% [wt/vol] acrylamide) gels.

A single precipitin arc was detected in immunoelectrophoresis experiments involving a cell-free extract of *V. splendidus* II strain 2 reacted with the antiserum to the alkaline phosphatase from this strain (anti AP-S). When a cell-free extract of *V. harveyi* strain 392 was reacted with antiserum to the alkaline phosphatase from this strain (anti AP-H), a second faint precipitin arc was observed which was traced to the antiserum from a single rabbit. The second precipitin band was not observed when purified enzyme was used instead of the crude extract. Since this minor contaminating antibody did not affect the results of the microcomplement fixation experiments, the antiserum was pooled with antisera from the other two rabbits and used in all subsequent studies.

Microcomplement fixation experiments. Representative results of the microcomplement fixation experiments with anti AP-S and anti AP-H are presented in Fig. 1 and 2, respectively. The titers with the homologous antigens were 1:34,000 for the former and 1:31,000 for the latter antiserum and represent 0 ImD in Fig. 1 and 2. No changes in titers were observed when purified enzymes rather than cell-free extracts were used as the antigen (data not shown). ImD values for the alkaline phosphatases from strains of *Vibrio* relative to the alkaline phosphatases from *V. splendidus* II strain 2 and *V. harveyi*

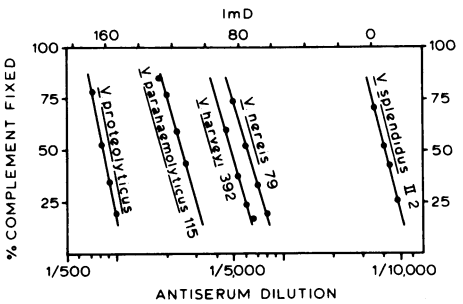

FIG. 1. *Relation of the percent complement fixed at equivalence to the log of the antiserum dilution. The antiserum used was to the alkaline phosphatase of V. splendidus II strain 2.*

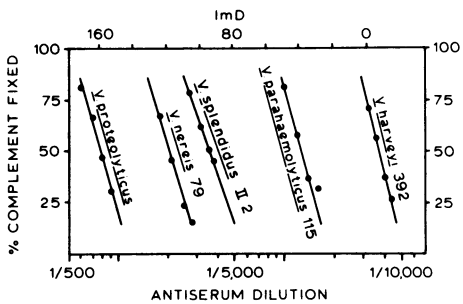

FIG. 2. *Relation of the percent complement fixed at equivalence to the log of the antiserum dilution. The antiserum used was to the alkaline phosphatase of V. harveyi strain 392.*

strain 392 are presented in Table 3. With the exception of *V. splendidus* I and II, all of the strains in each of the remaining species or groups encompassed a narrow range of ImD values, as is indicated by standard deviations equal to or less than ±8 ImD units. The reciprocal values between the two reference strains were 92 and 105 ImD units (average value of 99).

Groups of strains established on the basis of similarities of their alkaline phosphatases are presented in Fig. 3. In this figure, the alkaline

TABLE 3. *ImD values of alkaline phosphatases from different marine strains of Vibrio relative to V. splendidus II and V. harveyi*

Species, biotype, or group	ImD value (mean ± SD)[a]	
	V. splendidus II strain 2	*V. harveyi* strain 392
V. splendidus II		
2	0 (23 ± 15)	105 (104 ± 5)
8	26	95
10	17	103
12	36	108
14	34	107
V. splendidus I		
378	7 (19 ± 16)	105 (107 ± 3)
NCMB 1[b]	30	109
Strain 16	31	109
Strain 76	72 (72 ± 0)	95 (95 ± 0)
Strain 142	72	95
V. nereis		
79	85 (83 ± 2)	127 (125 ± 2)
80	83	123
82	81	126
V. harveyi		
72	ND[c] (93 ± 2)	13 (14 ± 8)
74	90	20
130	93	14
334	95	14
355	ND	22
392	92	0
Strain 331	100 (102 ± 3)	14 (14 ± 0)
Strain 333	105	14
V. campbellii		
18	101 (99 ± 4)	19 (16 ± 2)
28	102	14
34	98	15
40	92	15
58	ND	16
60	100	14
Strain 84	104 (104 ± 0)	74 (75 ± 1)
Strain 85	104	75
V. natriegens		
107	117 (117 ± 3)	79 (79 ± 2)
108	ND	78
109	120	79
110	ND	76
111	114	81
V. vulnificus		
320	ND (119 ± 3)	98 (98 ± 1)
321	122	99
324	117	99
329	118	96
V. alginolyticus		
86	ND (127 ± 4)	46 (48 ± 3)
90	123	45
93	130	47
120	134	47
122	125	52
BML-1	125	53
V. parahaemolyticus		
113	124 (125 ± 2)	46 (44 ± 2)
115	122	47
117	125	44
205	126	42
279	ND	43
ATCC 27519[d]	126	43
V. proteolyticus		
145	167	168

[a] SD, Standard deviation.
[b] NCMB, National Collection of Marine Bacteria.
[c] ND, Not determined.
[d] ATCC, American Type Culture Collection.

phosphatases from the strains in Table 3 are fixed in two-dimensional space by their ImD values relative to the two reference strains. The points are, however, free to rotate around the vertical axis represented by a line of 99 ImD units between the two reference antigens.

Ouchterlony double-diffusion experiments. The results of the immunodiffusion experiments which tested the alkaline phosphatases in cell-free extracts against anti AP-S and anti AP-H are presented in Fig. 4 and 5, respectively. The interpretation and organization of the data are according to Gasser and Gasser (15) and London (24). With the exception of *V. splendidus* I and II and strain 16, pairwise comparisons of strains within a species or between closely related species gave confluent precipitin bands, indicating identity or apparent identity of antigenic determinants. When a single spur was detected, an arrow is used to indicate a reaction of partial identity, the strain sharing the greater number of antigenic determinants with the reference alkaline phosphatase being designated by the direction of the arrow. A cross indicates that a double spur (reaction of partial or complete nonidentity) was observed. In this case, few or none of the antigenic determinants in the reference alkaline phosphatase were shared by the two heterologous antigens. The order of the species and groups in Fig. 4 and 5 is based primarily on decreasing numbers of spurs formed over the heterologous antigens (reflect-

ing increasing antigenic divergence from the reference alkaline phosphatase) and roughly correlates with an increase in ImD values.

The identity groups in Fig. 4 and 5 have unique spurring patterns which allow their differentiation from one another. The closely related species giving identity reactions with anti AP-S (Fig. 4) were (i) *V. harveyi* and *Vibrio campbellii* and (ii) *Vibrio alginolyticus* and *Vibrio parahaemolyticus*. With anti AP-H (Fig. 5), identity reactions were obtained with (i) *V. harveyi* and *V. campbellii* and (ii) *V. splendidus* I and II and strain 16. The alkaline phosphatases from strains of *V. splendidus* I and II, as well as strain 16, did not give reactions of identity with anti AP-S. The order of antigenic divergence established by their spurring patterns (data not shown) correlated with the ImD values in Table 3. The relatively broad range of ImD values as well as the heterogeneity observed in the Ouchterlony double-diffusion patterns indicated that their alkaline phosphatases were more diverse than would be expected for strains from a single species or biotype. Strains 331 and 333 gave a reaction of identity with the closely related species *V. harveyi* and *V. campbellii* when tested with anti AP-H. With anti AP-S, the same two strains gave a reaction of nonidentity with *V. harveyi* and one of identity with *V. campbellii*, suggesting that their alkaline phosphatases are more closely related to the enzymes from the latter species (results not included in Fig. 4 and 5). The identity groupings delineated by the Ouchterlony double-diffusion results are consistent with the arrangement of the strains presented in Fig. 3. The antigenic similarity of the strains within an identity group places constraints on their rotation around the vertical axis joining the two reference antigens in Fig. 3.

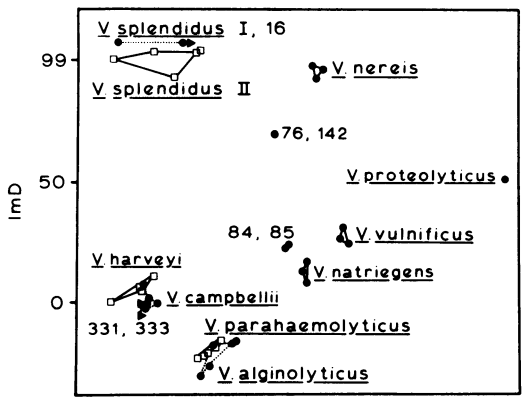

FIG. 3. *Amino acid sequence divergence (expressed as ImD) of alkaline phosphatase from species, biotypes, and groups of marine Vibrio relative to the reference enzymes from V. splendidus II strain 2 and V. harveyi strain 392. Symbols represent individual strains; those joined by lines are part of a single species, biotype, or group.*

Miscellaneous strains. The range of specific activities of alkaline phosphatase in cell-free extracts used in the microcomplement fixation experiments was 0.9 to 33. Strains of the following species had specific activities below 0.015 (number in parentheses indicates the number of

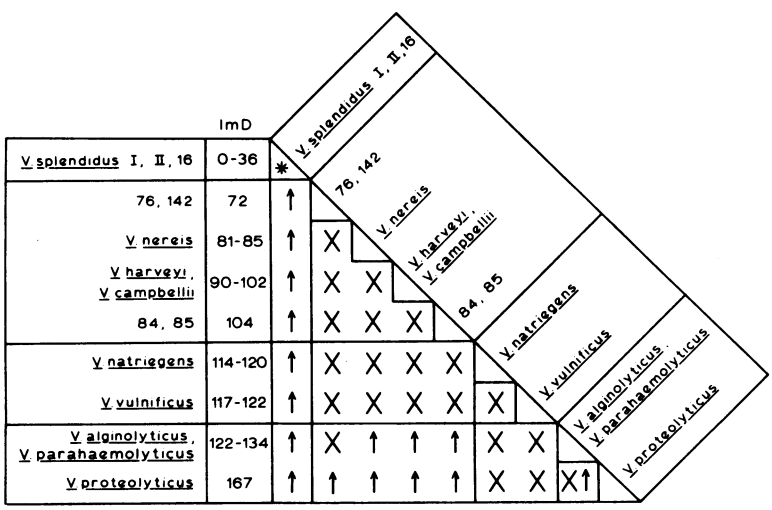

FIG. 4. *Summary of the results of Ouchterlony double-diffusion experiments with anti AP-S. Strains of species, biotypes, or groups giving identical spurring patterns with the other strains tested are grouped together. (*) Although V. splendidus I and II and strain 16 are presented together, they did not all give reactions of identity with each other; (↑) reaction of partial identity, arrow indicates dominant antigen; (×) reaction of nonidentity. (For further explanation see text.)*

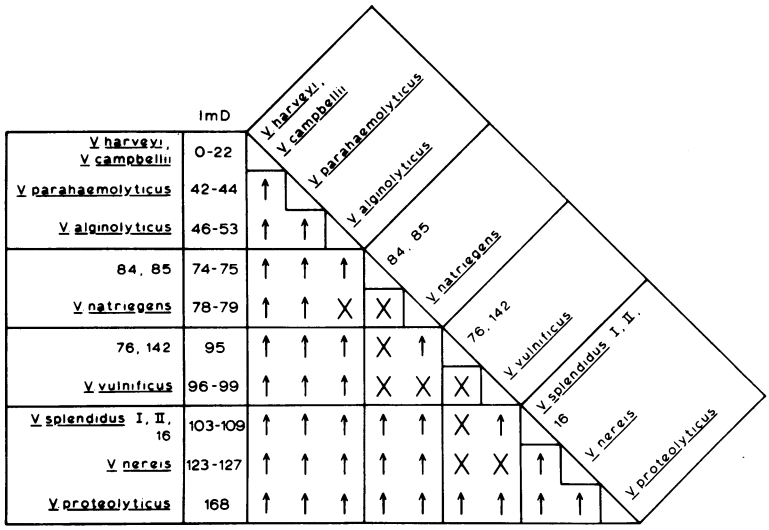

FIG. 5. *Summary of the results of Ouchterlony double-diffusion experiments with anti AP-H. For explanation of symbols see legend to Fig. 4.*

strains tested; for strain numbers consult M. J. Woolkalis, Ph.D. thesis, University of California, Davis, 1981): *V. cholerae* (24), *V. metschnikovii* (6), *Vibrio fischeri* (10), *Vibrio anguillarum* I (8) and II (5), *Vibrio fluvialis* I (9) and II (3), and *Vibrio pelagius* I (7) and II (4). Attempts to partially purify the putative alkaline phosphatase from cell-free extracts with specific activities of 0.010 to 0.015 were unsuccessful. Two strains of *V. splendidus* I and two strains of *V. harveyi* also lacked alkaline phosphatase. Strains 94 and 95, which are marine vibrios from group E-3 (5), and *E. coli* ATCC 11775-2 had specific activities of 1.9, 2.8, and 1.1, respectively, but showed no cross-reactivity in immunodiffusion experiments. This was also the case when the alkaline phosphatase from strain 95 was tested by the microcomplement fixation technique (ImD > 180).

DISCUSSION

The results of these studies show that species related by less than 62% DNA homology (29) are readily distinguishable by differences in their alkaline phosphatases. This conclusion is based on the groupings established by the ImD values of the alkaline phosphatases relative to the two reference antigens (Fig. 3) as well as on their characteristic spurring patterns in Ouchterlony double-diffusion experiments (Fig. 4 and 5). The exceptions to the latter are *V. parahaemolyticus* and *V. alginolyticus,* which have a DNA homology of 65% but differed in two spurring patterns (Fig. 5), and strain 16, which was not differentiated from *V. splendidus* II (Fig. 4 and 5) even though its DNA homology to this biotype is 56% (29).

A plot of the ImD values for heterologous alkaline phosphatases versus their corresponding DNA homologies is given relative to both *V. splendidus* and *V. harveyi* in Fig. 6. The high correlation coefficient (−0.89; −0.90 without *Vibrio proteolyticus*) indicates that a change in the amino acid sequence of alkaline phosphatase is approximately proportional to a change in percentage of DNA homology (a measure of the similarity of the total genome). A comparison of the ImD values of alkaline phosphatase and superoxide dismutase (S. S. Bang, L. Baumann, M. J. Woolkalis, and P. Baumann, manuscript in preparation) relative to *V. splendidus* II strain 2 (Fig. 7) established that the sequence change of one enzyme is proportional to the sequence change of the other (correlation coefficient = 0.92; 0.94 without *V. proteolyticus*). A major deviation from congruence in the comparisons shown in Fig. 6 and 7 occurred with the single available strain of *V. proteolyticus,* which has a higher ImD (167 and 168) than would be expected from DNA homology or the amino acid sequence divergence of its superoxide dismutase from the reference strain. We have no explanation for these findings. From Fig. 7 it is apparent that a change of 10 ImD units of alkaline phosphate corresponds to a change of about 2.2 ImD units of superoxide dismutase. Previous studies of superoxide dismutase and glutamine synthetase (2) have indicated that 2.2 ImD units of superoxide dismutase corresponds to about 0.6 ImD units of glutamine synthetase. The amino

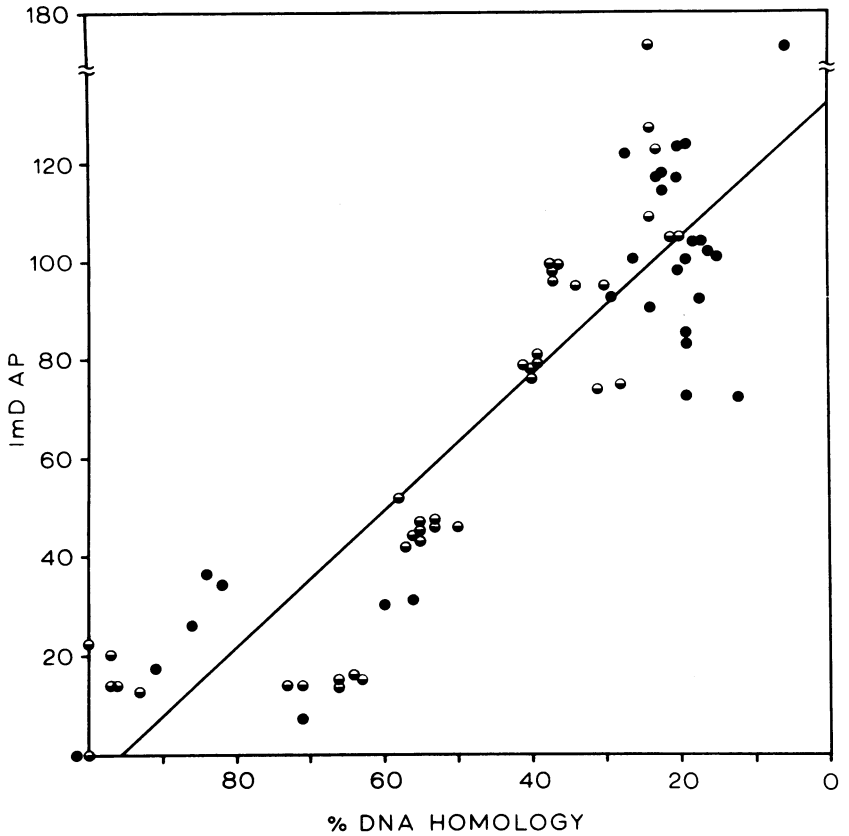

FIG. 6. *Relation of ImD of alkaline phosphatase (AP) to percentage of DNA homology relative to* (●) *V. splendidus II strain 2 and* (◒) *V. harveyi strain 392. Line obtained by linear regression analysis (correlation coefficient = −0.89; 70 comparisons). Data for DNA homology are from reference 29.*

acid sequence of alkaline phosphatase is, therefore, the least conserved of these three enzymes. Congruence between alkaline phosphatase and glutamine synthetase has also been noted among species from several genera to the *Enterobacteriaceae* (4, 12).

The results of this study support the view that evolution in marine species of *Vibrio* is primarily vertical and not horizontal. This conclusion is based on the congruence between the sequence changes of alkaline phosphatase, superoxide dismutase (Fig. 6), and DNA homology (Fig. 7). The latter extends the evidence for vertical evolution of the genes coding for alkaline phosphatase and superoxide dismutase as well as glutamine synthetase and rRNA (40) to a major portion of the bacterial genome and implies that most of the bacterial genome is not exchanged between species in the course of their evolution. A similar conclusion has recently been derived from a study of evolution in the fluorescent pseudomonads (9a). An ultimate verification of this conclusion will necessitate the sequence determination of a large number of informational molecules. These findings are not inconsistent with the potential of extrachromosomal elements to transfer genetic material between species, since the genes transferred appear to be restricted to relatively simple, functionally self-contained, dispensable properties which are advantageous to a bacterial population under certain environmental conditions (7, 9). The bulk of the bacterial genome, which is composed of genes coding for biosynthetic and primary energy-yielding pathways (1), does not appear to be exchanged between species in nature. This is probably due to the complex interdependence of many of these essential properties and the consequent lack of selective conditions necessary for their establishment and predominance in a bacterial population. Such selective conditions may be artificially established by genetic manipulation in the laboratory, but their relevance to genetic exchange in natural populations is difficult to assess.

Our studies do not address the problem of

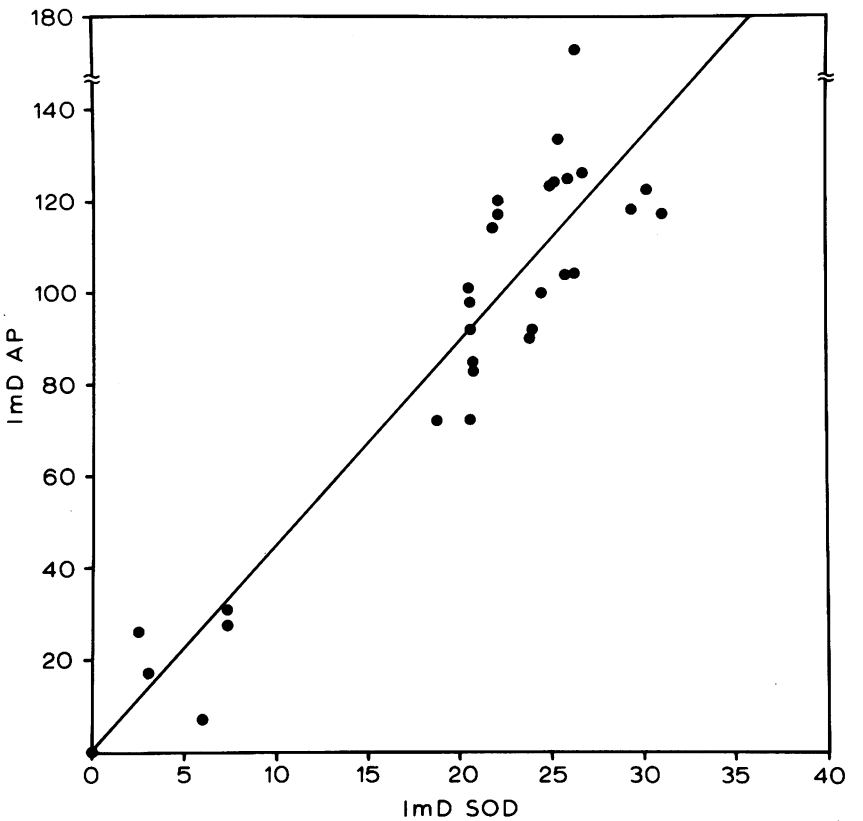

FIG. 7. *Relation of ImD of alkaline phosphatase (AP) to ImD of superoxide dismutase (SOD) relative to V. splendidus II strain 2. Line obtained by linear regression analysis (correlation coefficient = 0.92; 30 comparisons). Data for superoxide dismutase are from Bang et al. (in preparation).*

genetic exchange within a species. However, in view of the evidence of vertical evolution of species, it is interesting that a recent study of the electrophoretic variability of 20 enzymes from 109 clones of *E. coli* suggests that genetic exchange among strains in natural populations of this species, if present, occurs at a very low frequency (32).

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