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The sodium cycle. III. *Vibrio alginolyticus* resembles *Vibrio cholerae* and some other vibriones by flagellar motor and ribosomal 5S-RNA structures

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An electron microscopic study of the basal bodies of the *Vibrio albinolyticus* flagellum revealed a four-disc structure. The diameters of the two discs localized closer to the cytoplasmic membrane proved to be about 2-fold shorter than those of the two others. In this respect the basal body of *V. alginolyticus* resembles very much that of *V. cholerae* described by Ferris and co-workers. The sequence of the *V. alginolyticus* ribosomal 5S-RNA showed that it is similar to those of *V. cholerae*, *V. harveyi* and some other vibriones. On the basis of the 5S-RNA sequences, a dendrogram of prokaryotes is presented. It confirmed the suggestion that *V. alginolyticus* is a typical representative of *Vibrionaceae* rather than a 'monster' greatly differing from other vibriones. Possible evolutionary relation of various bacterial species possessing the primary Na^+ pumps is discussed.

Introduction

The two preceding papers [1,2] summarized data indicating that *Vibrio alginolyticus* employs Na^+ instead of H^+ as the coupling ion. In fact, this microorganism represents the first example of a living cell using the sodium cycle to couple an energy-releasing process (respiration) to the performance of all the three main types of the membrane-linked work, i.e., chemical, mechanical and osmotic.

The question arises: what place does *V. alginolyticus* take in the kingdom of bacteria? Is it a 'monster' or a rather usual member of, say, the *Vibrionaceae* family? There are some indications that the latter alternative is more probable. As it was found in this group, the properties of the *V. harveyi* motility system are basically the same as those of *V. alginolyticus* [3]. According to Tokuda and Unemoto [4], Na^+-motive respiration is also inherent in *V. costicola*.

To obtain further information related to the above problem we decided to compare *V. alginolyticus* with other eubacteria by studying two completely different structural parameters, namely, the flagellar motor structure and the nucleotide sequence of ribosomal 5S-RNA. The former aspect

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Abbreviations: $\Delta\bar{\mu}_{H^+}$, $\Delta\bar{\mu}_{Na^+}$, electrochemical gradients of H^+ and Na^+, respectively; $\Delta\psi$, transmembrane electric-potential difference; ΔpH and Δp*Na*, transmembrane differences in concentrations of H^+ and Na^+, respectively; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; HQNO, 2-heptyl-4-hydroyquinoline *N*-oxide; PCB^-, phenyldicarbaundecaborane; TPP^+, tetraphenylphosphonium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfphonic acid; Taps, 3-{[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]amino}-1-propnaesulphonic acid; Ches, 2-(cyclohexylamino)ethanesulphonic acid.

appeared interesting to us in connection with the recent work by Ferris et al. [5] who described basal body structure of the notorious representative of the same family, *V. cholerae,* and compared it with that of some other eubacteria. As to the 5S-RNA structure, it seems to be one of the most realistic bases for constructing an evolutionary dendrogram of living organisms. In fact, ribosomal RNA molecules are very conserved evolutionally and are ubiquitous among all the modern types of life. 5S-RNA is rather short and hence may easily be sequenced. The 5S ribosomal RNA sequence comparison is already used for elucidating evolutionary relations [43,44]. We employed this approach to reveal the phylogenetic position of *V. alginolyticus.*

The results of our study showed that *V. alginolyticus* is quite similar to *V. cholerae* with respect to both the flagellar motor and the 5S-RNA structure. No evidence for any special position of *V. alginolyticus* in the dendrogram of prokaryotes was obtained.

Methods and Materials

To isolate basal bodies of the *V. alginolyticus* flagellae, 3 l of the growth medium was centrifuged at $6000 \times g$ for 10 min. The pellet was suspended in 70 ml of distilled water and stirred for 1 h at 4°C.

Then the following consecutive additions were made: (i) 7 ml 1 M Tris-buffer (pH 7.8), (ii) 2 ml 2.5% lysozyme in 0.1 M Tris-buffer and 0.2 M NaCl, (iii) 6 ml 0.1 M EDTA and 0.1 M Tris-buffer and (iv) Brij-58 to a final concentration of 1%. The mixture was incubated for 1.5 h at 30°C. Thereafter, it was supplemented with 7.5 ml 20% Triton-X-100 and stored overnight at 4°C. Then 0.8 ml 1 M $MgSO_4$ and 30 mg DNAase-1 were added. After 20 min incubation at 30°C, the mixture was diluted with 3 vol. of cold 0.1 M Tris (pH 7.8), containing $5 \cdot 10^{-4}$ M EDTA (Tris-EDTA-buffer). The final solution was supplemented with saturated $(NH_4)_2SO_4$ to 25% of saturation, stored overnight at 4°C and centrifuged at $12000 \times g$ for 30 min. The sediment was dissolved in 100 ml of Tris-EDTA buffer with 0.1% Triton-X-100 and dialyzed against the same solution during the night at 4°C. The dialyzate was centrifuged at $105000 \times g$ for 1.5 h in tubes containing two 2 ml layers of sucrose solutions (60% and 20% for the lower and upper layers, respectively). After centrifugation, the upper sucrose layer was dialyzed against 0.1% Triton X-100 in the Tris-EDTA buffer during the night at 4°C and then re-centrifuged for 6.5 h at $105000 \times g$ in a sucrose gradient composed of 60%, 53%, 50%, 47%, 44%, 41%, 38%, 36%, 34% (one portion each) and 5% (10 portions) sucrose. The fraction of flagellae was localized in 53% sucrose. This fraction was dialyzed against 0.1% Triton-X-100 in a Tris-EDTA buffer for 24 h at 4°C. The storage of flagellae in water for 2–3 days resulted in dissociation of flagellar fibrils, the basal bodies remaining intact.

Electron microscopy of the basal bodies was carried out in a Hitachi 11B microscope by the negative contrasting method involving 1% uranyl acetate.

To prepare cross-sections of the *V. alginolyticus* cells, the bacteria were fixed with 2.5% glutaraldehyde and then with 1% osmium tetroxide, or as it was described by Kellenberg et al. [7]. Dehydration of bacteria was carried out with alcohols of increasing concentrations. The 70% alcohol was supplemented with uranyl acetate. The dehydrated preparation was embedded in Epon 812.

The sections were prepared by using an LKB-III Ultramicrotome, treated with lead citrate according to Reynolds [7] and viewed in Hitachi 11B, 12 or 700 H electron microscopes.

To isolate ribosomal 5S-RNA, the following procedure was employed. Nucleic acids were obtained from the *V. alginolyticus* cells by means of extraction with phenol and 0.5% sodium dodecylsulfate at 60°C [8]. Then the ribosomal RNA was separated from tRNA and DNA by using a CF-11 column [9]. The 3′-end of RNA was labelled with [^{32}P]cytidine (3′,5′)diphosphate by means of T4 bacteriophage RNA-ligase [10]. 5S-RNA was isolated by electrophoresis in 8% polyacrylamide gel containing 7 M urea [11]. The 5S-RNA zone was radioantographically localized, removed and eluated with a solution of 0.5 M ammonium acetate/10 mM $MgCl_2$/0.1% sodium dodecylsulfate. To sequence 5S-RNA, partial degradation according to Peattie [12] was employed. The degradation products were analyzed in 12% and

6% polyacrylamide gels which were 500 × 300 × 0.19 mm and 1000 × 300 × 0.19 mm, respectively.

Results

Basal body of the V. alginolyticus flagellum

An electron microscopic study of the *V. alginolyticus* cross-sections showed that the single membrane-coated flagellum of this microorganism is attached to the cell in such a way that the outer cell membrane continues to the flagellar membrane, whereas the central basal body rod crosses the outer membrane and periplasm reaching the inner cytoplasmic) membrane (Fig. 1 A–D). Four discs were identified in the basal body. The two discs proximal to the cytoplasm (M and S) proved to be smaller in diameter than the two others (P and L), as it was revealed by an electron microscope study of the isolated basal bodies (Fig. 1E). In the same micrograph it is seen one more disc-like structure of an intermediate diameter, localized between M,S pair and P,L pair. Further study is necessary to reveal whether it belongs to the basal body or represents a membrane fragment.

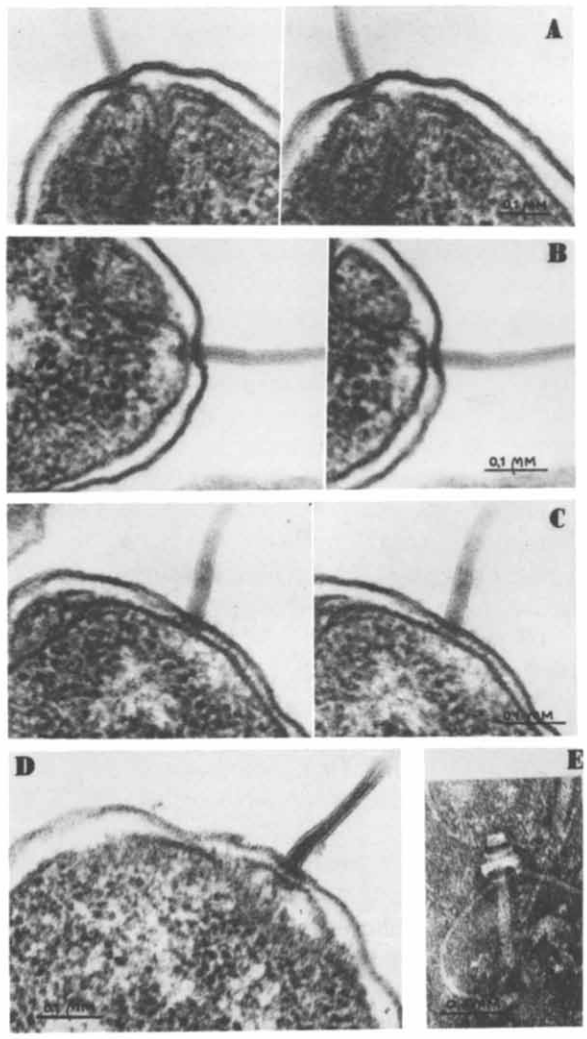

Fig. 1. Structure of the Na^+ motor of *V. alginolyticus*. (A–D), cross-sections of the bacterial cell. (E), isolated basal body of the flagellum. Magnification, ×80000. The thicknesses of sections in A, B and in C, D were 100 nm and 50 nm, respectively. A–C, stereopairs.

In Table I the diameters of the discs in several bacterial species measured in this group and Ferris et al. group [5] are compared. One can see that the ratio of the diameter of the L disc to that of the M disc varies from 2.2 in *V. alginolyticus* down to 0.6 in *Aquaspirillum serpens*. It should be noted that these ratios are practically the same in *V. alginolyticus* and *V. cholerae*.

The primary structure of ribosomal 5S-RNA

The results of the sequence analysis of the *V. alginolyticus* 5S-RNA are given in Fig. 2. For comparison, some other 5S-RNA structures are shown. The sequences of *Vibrionaceae* and of other bacteria are from MacDonell and Colwell [13] and Erdmann et al. [14], respectively. The comparison was made after a unified consensus arrangement of the sequence (see Ref. 14). It is seen that the 5S-RNA sequence from *V. alginolyticus* is very similar to those from *V. cholerae* and *V. harveyi*.

To reveal evolutionary relations of *V. alginolyticus* to other bacteria, a dendrogram shown in Fig. 3 was constructed (for detailed description of the algorhythm employed, see Ref. 15).

Briefly, the 'evolutionary work' (defined as the proportion of back mutations leading to an evolutionary conservation of the sequence) that underlies mutations in a given sequence element (nucleotide position of a consensus structure) was accounted, and the value of 'weight' for ith sequence element (W_i) was calculated. This calculation was performed on the basis of analysis of conservativity of sequence elements in the representative array of sequences. The distance D between species j and k was calculated as: $D_{j,k} =$

```
1  --GUUUGGCGAUUAUAGCGUUUUGGACCCACCUGAUU-UCCAUGCCGAACUCAGAAGUGAAACG
2   U C        CC   C  A                  CU      U
3   C CC       CC                 U       C  -    U
4   U C        CC          A    CAAA       C  -  U             U
5   U CC      GCCG        CGG    U         CC -
6  UG CC    U G C  U     GGC C  AA     C    C  -     C          G CC       GA
7   GCCCACC   G C C  U  AGCG   CAA     C GAC -U    UU       C G      U   G C
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1  AAAUAGCGCC--GAUGGUAGUGUGG-GGCUUCC-CCAUGUGAGAGUAGGA-CAUCGCCAGGCAU---
2    U                                C-                            U
3          U                    U     C-              U             C-
4  U                            UC    C-
5  CCG                          UC    C-     C        G A CU
6  GCCCU        A       C  C - UCU AAGG- G  G          U  GC         C
7  GCUC-A  UUAGUGG   CC    GAUACCG GAGGAUCC C-  CCCCACUAAGCU GG U GG UUU
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Fig. 2. The nucleotide sequence of ribosomal 5S-RNA from: 1. *V. alginolyticus* (this study); 2. *V. harveyi* [13]; 3. *V. cholerae* [13]; 4. *Photobacterium phosphorum* [13]; 5, *E. coli* [14]; 6, *Rhodospirillum rubrum* [14]; 7, *Sulfolobus sulfataricus* [14]. A complete *V. alginolyticus* sequence is shown. For other bacteria, only those nucleotides that differ from *V. alginolyticus* are indicated.

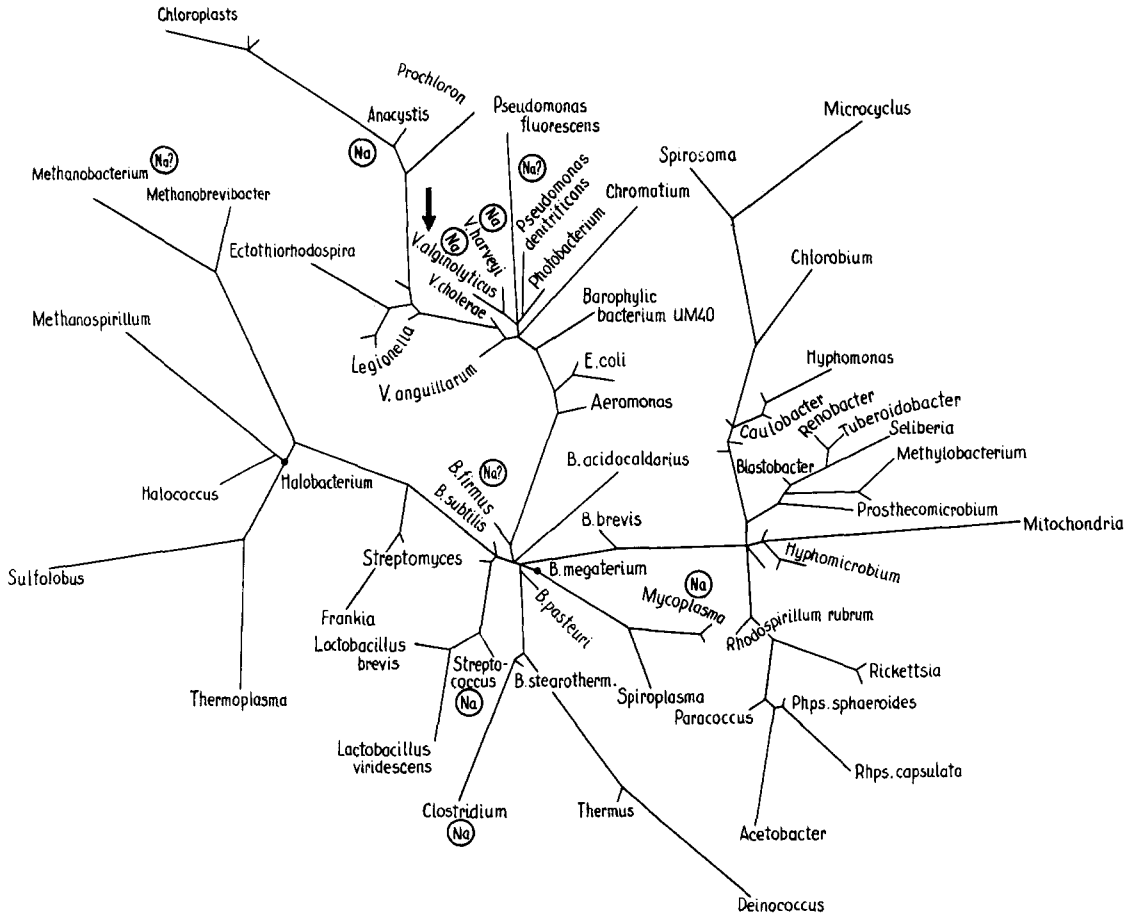

Fig. 3. Dendrogram of the bacterial evolution constructed with the aid of the 5S-RNA sequences. (Na), bacteria possessing Na^+ pumps instead of or in addition to H^+ pumps.

TABLE I

THE STRUCTURE OF BASAL BODIES OF FLAGELLAR MOTORS

Diameters of the basal body discs, measured in this study and in that of Ferris et al. [5] are given.

Disc	Diameter (nm)					
	Vibrio alginolyticus	*Vibrio cholerae*	*Campylobacter fetus*	*Aquaspirillum serpens*	*Escherichia coli* MS 1350	*Bacillus brevis* G-B P^+
L	33	23	39	18	21	–
P	33	23	39	21	21	–
S	15	11	26	28	18	20
M	15	11	26	31	18	20
L-to-M ratio	2.2	2.1	1.5	0.6	1.2	–

$\Sigma_i W_i$. Use of these 'weighted distances' allows to take into account the differences in the mutation rates at different sequence elements. Estimation of the phylogenetic tree topology was performed by the cluster method based on the 'weighted distances' matrix followed by the reconstruction of the sequences that correspond to the nodal points of the tree. The distances presented on the tree are proportional to the number of mutations.

As it is clear from the dendrogram in Fig. 3, *V. alginolyticus* looks like a typical representative the *Vibrionaceae* family. Again, a close relation of *V. alginolyticus* to *V. cholerae* was revealed.

Discussion

Taking into account the close evolutionary relation of *V. alginolyticus* and *V. cholerae* revealed by a study on the 5S-RNA sequence as well as on the flagellar motor structure, one may ask whether Na^+ is the coupling ion not only in *V. alginolyticus*, but also in *V. cholerae*.

It should be noted in this context that (i) the pH optimum of *V. cholerae* growth is shifted to the alkaline pH; (ii) this vibrio can survive at pH 10.0 and in 4% NaCl (some strains at pH 11.0 and in 8% NaCl), whereas acidification of the medium kills it [16]; and (iii) as it was already mentioned at least certain features of the Na^+ energetics are inherent in some other vibriones, i.e., *V. harveyi* [3]. Assuming that there is the Na^+ cycle also in *V. cholerae*, we may explain the role of the *V. cholerae* toxin for growth of this microorganism in intestine. It is known that this toxin causes a large-scale extrusion of the salt from tissues to the intestine lumen where *V. cholerae* lives. The high salt level may be favourable for the growth of *V. cholerae* at alkaline pH in the infected intestine provided that this vibrio, like *V. alginolyticus*, employes the Na^+ cycle. Such a possibility is now under study.

General conclusion

Thus the data presented in this series are consistent with the idea that *V. alginolyticus* employs Na^+ instead of H^+ as the coupling ion to survive at a low outer H^+ concentration. Judging from the flagellar motor structure and the 5S-RNA sequence, *V. alginolyticus* is a typical representative of *Vibrionaceae*, rather than a curiosity vastly different from other vibriones. It seems likely that certain other vibriones, including *V. harveyi* and perhaps *V. cholerae*, also possess Na^+-energetics.

There are indications in the literature that *Vibrionaceae* are not the only group of living organisms employing at least some components of the Na^+-cycle. As it was established by Dimroth and his group as well as by Buckel and Semmler, several species of anaerobic bacteria use Na^+ ions to couple some decarboxylation processes to the performance of chemical and/or osmotic work. Among them there are *Propionigenum modestum* [17], *Klebsiella aerogenes* [18–22], *Veilonella alcalensis* [23,24], *Acidaminococcus fermentans*, *Peptococcus aerogenes* and *Clostridium symbiosum* [25,26].

In anaerobic *Streptococcus faecalis* utilizing glycolytic ATP to energize the membrane, there are two ATP-splitting membrane enzymes, i.e., H^+-

ATPase and Na^+-ATPase. The latter is greatly activated under conditions where H^+-energetics is impossible due to (i) mutation in H^+-ATPase or (ii) addition of a protonophore to the growth medium [27]. In the same bacterium, it was found that H^+-ATPase is suppressed at alkaline pH [28]. A related observation was made when facultative alkalophile *Exiguobacterium aurantiacum* was studied. It was shown that here there is a primary Na^+ pump using glycolytic energy to export Na^+ [29,30]. There are some indications that Na^+-ATPase in *Mycoplasma mycoides* [31,32] and *Acholeplasma laidlawii* [33–35].

Among respirers, a halotolerant bacterium Ba_1 seems to possess Na^+-motive succinate oxidase [36,37]. A possible energy-coupling role of Na^+ in cyanobacterium *Oscillatoria brevis,* methanogenic bacteria, *Pseudomonas stutzeri* and alkalophilic bacilli, including *Bacillus firmus* was discussed in the preceding papers [1,2]. In the outer membrane of the animal cell, co-operation of the Na^+,K^+-ATPase or of the recently discovered Na^+-ATP [38–40] and Na^+, solute-symporters can also be regarded as a version of the Na^+-cycle, transducing the ATP energy into the osmotic work [41,42].

In Fig. 3 microorganisms possessing the primary $\Delta\bar{\mu}_{Na}$-generators are indicated by symbol (Na). It is seen that they are localized along the line leading from *Clostridium* to vibriones and *Pseudomonas* and cyanobacteria, with branches toward *Mycoplasma, Streptococcus* and possibly to methanobacteria. (It should be stressed that the presence of $\Delta\bar{\mu}_{Na}$-generator(s) does not exclude existance of $\Delta\bar{\mu}_{H}$-generator(s) in the same membrane as it is the case in *Streptococcus faecalis*).

It is obvious that examples of sodium energetics are still less numerous than those of the protonic cycle. However, we should take into account that the pioneering studies on the role of Na^+ as the substitute for H^+ were undertaken only recently. The great taxonomic variety of organisms employing the Na^+-cycle points to the ubiquitous distribution of this novel type of membrane-linked energy transductions.

If this is the case, *V. alginolyticus* is not a 'unicum', but rather a message from a yet unknown but possibly extensive sodium world.

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