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1 November 1991

Dr. Samuel Kaplan
Editor in Chief, Journal of Bacteriology
Journals Division
American Society for Microbiology
1325 Massachusetts Avenue, N.W.
Washington, DC 20005-4171

Dear Dr. Kaplan:

We are pleased to submit the enclosed manuscript entitled "Phylogenetic Relationship of Chemoautotrophic Bacterial Symbionts of *Solemya velum* Say (Mollusca: Bivalvia) Determined by 16S Ribosomal RNA Gene Sequence Analysis" for consideration for publication in the Journal of Bacteriology. Included are three complete copies of the manuscript with 4 figures and 1 table. The originals for Figures 1 and 3 are available upon request.

We suggest the following investigators who are working in the area of ribosomal molecular phylogeny or chemoautotrophic symbiosis as possible reviewers of this manuscript:
Dr. Edward DeLong, Woods Hole Oceanographic Institution, Redfield Building, Woods Hole, MA 02543, (508) 457-2000, X2948.
Dr. Douglas Nelson, University of California, Bacteriology Dept., Davis, California 95616, (916) 752-6183.
Dr. Norman Pace, Indiana University, Dept. of biology, Jordan Hall 138, Bloomington, IN 47405, (812) 855-6152.

Thank you for consideration of our manuscript. If any further information would be helpful, please do not hesitate to contact me.

Yours sincerely,

Colleen M. Cavanaugh
Assistant Professor
Dept. of Organismic and Evolutionary Biology

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Dr. Colleen M. Cavanaugh
Department of Organismic and
Evolutionary Biology
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11 March 1992
Month: May 92
Volume: 174
Issue: 10
Article: JB 1468-91

Authors: Jonathan A. Eisen, Steven W. Smith, Colleen M. Cavanaugh

Title: Phylogenetic Relationships of Chemoautotrophic Bacterial Symbionts of *Solemya velum* Say (Mollusca: Bivalvia) Determined by 16S rRNA Gene Sequence Analysis

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Agreed to by author.
(Signed) Colleen M. Cavanaugh
Date 16 March 1992

(Author: Please sign on behalf of all co-authors. Return the agreement to ASM in the enclosed envelope.)

JOURNAL OF BACTERIOLOGY
A publication of the American Society for Microbiology

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January 15, 1992

Dr. Colleen M. Cavanaugh
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16 Divinity Avenue
Cambridge, MA 02138
U.S.A.

Dear Dr. Cavanaugh:

Re: JB 1468-91
Phylogenetic relationship of chemoautotrophic bacterial symbionts of Solemya velum Say (Mollusca: Bivalvia) determined by 16S ribosomal RNA gene sequence analysis

Your paper received somewhat mixed reviews, although both reviewers agree that it is too long. Beyond that, one thinks it is acceptable and the other that it is only a marginal contribution to the literature. I will agree to accept it, but only if the paper is shortened to Note format and length.

Please return three clean copies of the entire revised manuscript, including figures, and one copy of the original manuscript, with changes marked on it, as soon as possible. The revised manuscript should be accompanied by a letter stating your disposition of each of the reviewers' suggestions, item by item. If you wish to reject any or all suggestions, please state your reasons. The manuscript must be returned to me within two months, or it may be considered withdrawn.

I shall look forward to hearing from you.

Sincerely yours,

James D. Friesen, Ph.D.
Editor

JDF/abw
encl.

JE
Talk to you soon.
Cx

JOURNAL OF BACTERIOLOGY
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JAMES D. FRIESEN
Research Institute
The Hospital for Sick Children
555 University Avenue
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Manuscript no.: JB 1468-91 Date: November 26, 118 Reviewer no.: 1

Dear Reviewer: Please complete this form and return three copies with the manuscript. Scientific comments should be numbered and, where appropriate, the manuscript page number should be cited, so that revised manuscripts can be checked easily for adherence to your comments.

COMMENTS FOR THE AUTHOR'S CONSIDERATION:

The manuscript by Eisen *et al.*, presents a phylogenetic analysis of a *Solemya velum* symbiont based on a 16S rRNA sequences, and data indicating (but not proving) that the symbiont rRNA gene is single-copy. Overall the paper is a thorough one, but the important conclusion, the phylogenetic analysis, in essence is confirmatory of previous conclusions. Therefore, in this reviewer's opinion, the report does not belong in J. Bacteriol. Rather, a more specialized journal (e.g. IJSB) is the appropriate vehicle. Certainly the information should be published, but at this stage does not stand alone for J. Bacteriol.

Although the science is sound, the manuscript is overwritten. Introduction, Methods and Discussion in any revised version of the manuscript could be reduced ca. two-fold. Figs. 1 and 2 (gel of PCR product and predicted restriction map) could be eliminated with no loss of relevant documentation. The authors also should note the spelling of complementary, in regard to base-pairing sequences (e.g. in Methods).

JOURNAL OF BACTERIOLOGY
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Manuscript no.: JB 1468-91 Date: Nov. 26, 1991 Reviewer no.: 2

Dear Reviewer: Please complete this form and return three copies with the manuscript. Scientific comments should be numbered and, where appropriate, the manuscript page number should be cited, so that revised manuscripts can be checked easily for adherence to your comments.

COMMENTS FOR THE AUTHOR'S CONSIDERATION:

This paper is an important contribution and should be published. The data are sound but the Introduction and Discussion are over length and should be shortened.

The Introduction is overly broad in scope and too long. It could easily be cut by 1/3 to 1/2 with no damage to the paper. The authors are fundamentally adding a single 16S rRNA sequence to an already significant data base and establishing that a single gene copy probably exists in their symbiont. Therefore neither a listing of tube worm, oligochaete and bivalve genera hosting symbionts nor a 3/4 page description of the Soleyma symbiosis is called for.

Figure 1 should be deleted and the results simply stated in the text. PCR amplification is now routine. Figure 3 is a selected subset of all hybridization data and there were no differences observed in any comparison of different individuals. Thus, it seems difficult to argue for keeping Fig. 3. (To show bands resulting from incomplete digests is the only reason I can think of for keeping the figure.) Authors state that 9 different restriction enzymes were used (pg. 7). Patterns are shown in Fig. 3 for only 7 of these used singly. Band sizes resulting from the other two should be stated in the text. Simply stating in text all band sizes resulting from single and double digests would be the most compact approach. I did not find the stringency conditions of hybridization described in the Methods or Figure Legends. I am certain that the Maniatis reference offers a range of hybridization conditions.

Results pg. 10, line 14. Authors should refrain from attributing chemoautotrophic potential to Thiothrix nivea. This reviewer knows of no published demonstration of this assertion.

Results pg. 10, last 3 lines. Omit. The alternate affiliation of C. burnetti is not relevant.

The Discussion is overly long. This and the over length Introduction result in an excessive Literature Cited section. From the second paragraph of pg. 12 to the end of pg. 13 the authors deal with rRNA operon copy number in their symbiont and the possible

(over)

association of copy number with growth rate or relaxation of selective pressure. The essential points can be made in one carefully crafted paragraph. However, authors should mention the possibility that a large enough duplication could be present in S. velum genome to provide duplicate rRNA operons which would escape detection by their analysis. Given the number of restriction enzymes they employed and fragment sizes generated they could even estimate how different theoretically duplicated regions could be and still escape detection by the approach taken.

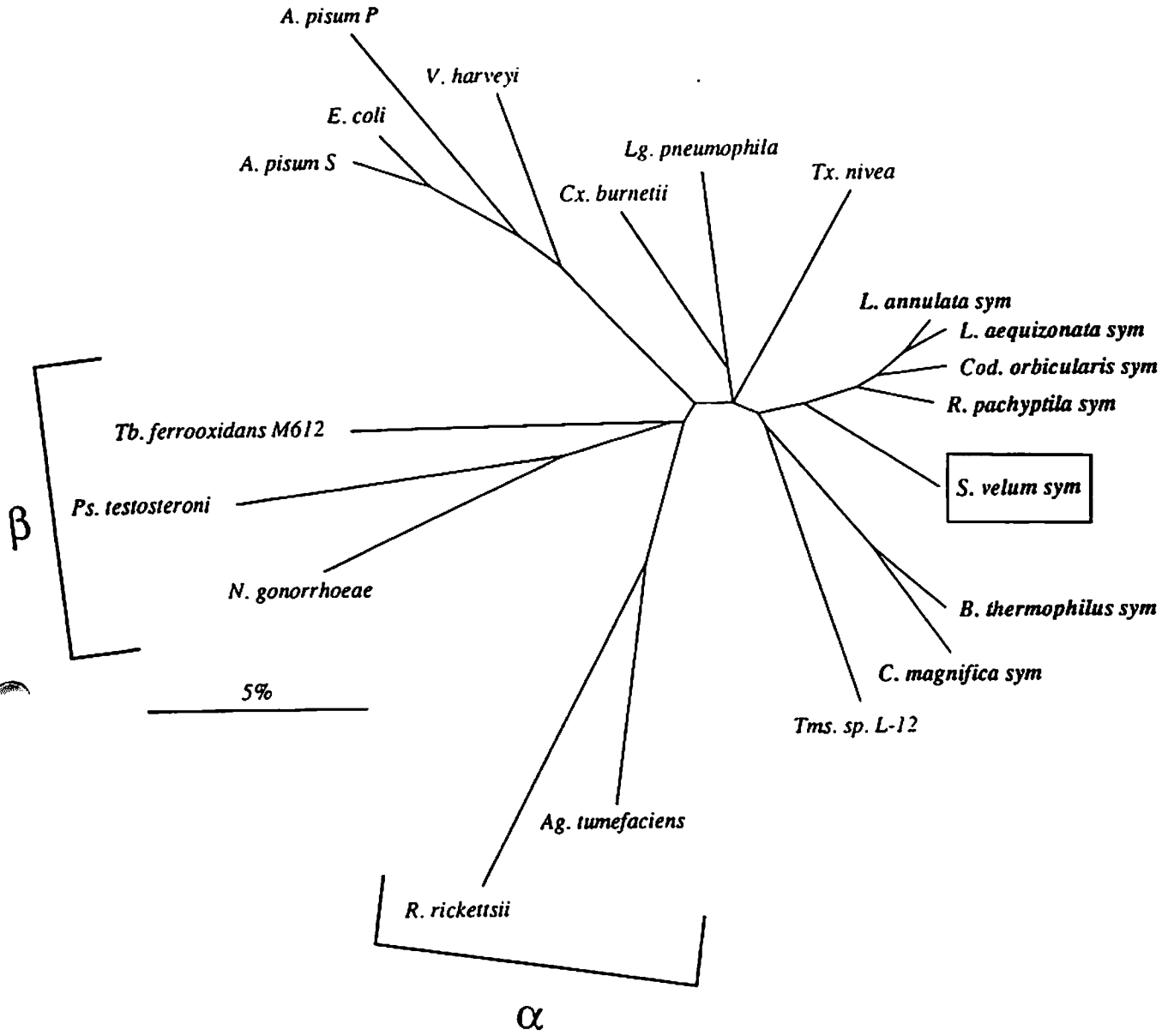
Pg. 14, line 9. Again, T. nivea is not a proven chemoautotroph.

Pg. 14, line 10 to the end of page. Drastically reduce or eliminate. The clustering of S. velum symbiont with C. burnetti/L. pneumophila seems to be over analyzed. The Proteobacteria contain most, but not all, of the traditional Gram negative bacteria (ref 62). (Spirochetes, green-sulfur bacteria, green-non-sulfur bacteria, and cyanobacteria are the other Gram negative groups.) There are obvious structural reasons why most pathogens and intracellular symbionts are favored to have a Gram negative wall structure and be non-photosynthetic. If this is this the authors' point it is much too general for tufs paper. Drawing developmental parallels between C. burnetti and S. reidi based on a 30 year old reference (ref 40) to C. burnetti engaging in "apparent de novo formation of bacteria from condensed granular material" is an over reach. The life cycle of C. burnetti, with its alternation of vegetative growth and spore generation is now well understood (Weiss, 1982 Ann Review of Microbiology). The S. reidi reference (ref 23) strikes this reviewer as muddled and it certainly does not differentiate between growth of symbionts from "spores" or de novo acquisition from the environment.

The PCR was used to amplify the 16S rRNA coding regions from the S. velum symbionts of

A modification of the PCR method of weisburg et al was used to amplify the 16S rRNA coding regions of the S. velum symbionts. (see results) DNA products... The amplified genes were ~~sequenced directly according to a modification of~~ (see methods) purified & sequenced directly. The complete gene was seq. completely in both directions from one animal (bases...) and partially (bases...) for two others. The DNA from 9 animals was submitted to restriction analysis. See methods...

Phylogeny...


A. pisum P
V. harveyi
E. coli
A. pisum S
Lg. pneumophila
Cx. burnetii
Tx. nivea
L. annulata sym
L. aequizonata sym
Cod. orbicularis sym
R. pachyptila sym
S. velum sym
Tb. ferrooxidans M612
Ps. testosteroni
N. gonorrhoeae
β
5%
B. thermophilus sym
C. magnifica sym
Tms. sp. L-12
Ag. tumefaciens
R. rickettsii
α

JB
JOURNAL of BACTERIOLOGY
Twice Monthly

ASM CONTROL NO. JB 1468-91 ASM ARTICLE NO. N (T = TEXT, N = NOTE, R = MINIREVIEW) DOCKET Byrd 6602

SECTION Genetics EDITOR Friesen

NUMBER OF SHEETS OF MANUSCRIPT: 18 NUMBER OF TABLES INCLUDED: 1

NUMBER OF FIGURES: 4 (4 pieces) NUMBER OF TONES: 2 pieces

RUNNING HEADS AND FOLIOS

LEFT PAGE RUNNING HEAD:

000 NOTES J. BACTERIOL.
AUTHOR(S)

RIGHT PAGE RUNNING HEAD:

VOL. 174, 1992 NOTES 000
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Dr. James D. Friesen
Editor, Journal of Bacteriology
Research Institute
The Hospital for Sick Children
555 University Avenue
Toronto, Ontario M5G 1X8
Canada

3 March 1992

Dear Dr. Friesen:

Enclosed is our revised manuscript [JB 1468-91] entitled "Phylogenetic relationship of chemoautotrophic bacterial symbionts of *Solemya velum* Say (Mollusca: Bivalvia) determined by 16S ribosomal RNA gene sequence analysis" [JB1468-91]. Included are three copies of the new manuscript including the figures. I have also enclosed the original plates for Figures 1 and 3.

We thank you for your suggestions and those of the reviewers. The paper has been shortened to Note format and length. I have also included a copy of the original manuscript, in which I have attempted to indicate the major parts which have been deleted and sections which have been moved. However, since the paper has been drastically shortened, I have also outlined these deletions on the attached sheets, along with our disposition of the reviewers' suggestions.

Thank you very much for consideration of our revised manuscript for publication in Journal of Bacteriology.

Sincerely yours,

Colleen M. Cavanaugh
Assistant Professor
Dept. of Organismic and
Evolutionary Biology

JB1468-91
J.A. Eisen, S.W. Smith, and C.M. Cavanaugh

Phylogenetic relationship of chemoautotrophic bacterial symbionts of Solemya velum Say (Mollusca: Bivalvia) determined by 16S ribosomal RNA gene sequence analysis

Length of the manuscript: The paper has been shortened from 13 pages text to 7 pages. Major sections which were deleted are crossed out in red on the original text. The title and abstract were not changed.

Specific deletions include:

INTRO: 2 out of 5 paragraphs

METHODS: Considerable detail, which can be found in references cited.

RESULTS: Section on Proteobacteria peripheral to the symbiont cluster, i.e., C. burnetti, T. nivea, and L. pneumophila.

DISCUSSION:

Section on rrn copy number cut to one paragraph.

Section on relationship of chemoautotrophic cluster to peripheral bacteria.

REFERENCES: have been reduced from 64 to 39.

TABLE- section describing how percent similarity and evolutionary distance are calculated was moved to table footnotes.

Format: Typical paper headings (Intro, etc.) have been replaced by topic headings. There is a general introduction to the paper. Then under each heading, typically there is a brief introduction followed by methods, results, and discussion for each topic.

Figures: Between the two reviewers, it was suggested to delete the first three figures. We think that they are an important part of the paper and should be retained for the following reasons.:

Figure 1. While PCR amplification is now routine, this is not just a PCR figure, but is showing that the 16S rRNA gene was amplified only from gill tissue, from a mixture of procaryotic and eucaryotic DNA, and not from the foot. This fig... could be quite small.

Figure 2. The restriction map is an important component of our method ... that the 16S gene amplified from gill DNA was indeed dominan... necessary to interpret the restriction analysis indicating that th... gene is single-copy. This figure could also be quite small.

Figure 3. This figure is important precisely because it does sho... pattern for different animals, supporting the sequence da... type of symbiont occurs in *S. velum.* Furthermore, the res... those predicted by the sequence, indicating we did ampli... the gill tissue.

Overall, we think Figs. 1 and 3 should be shown for the critique of ... these are not standard analyses from pure cultures, but rather PCR ... procaryotic and eucaryotic DNA, and restriction analysis of symbio... different individual clams. Figure 2, is an important model for desig... interpretting the restriction analyses.

...ure ... for confirming ... t in the gill, and ... e symbiont rRNA ... restriction

Reviewer No. 1.

1. The paper has been reduced almost by one half, notably the Introduction, Methods, and Discussion (see specific details above).

2. Figures 1 and 2- please see comments above.

3. Comple->imentary has been noted and changed.

Reviewer No. 2

1. The Introduction has been cut by 1/2. The section listing the other genera and the lengthly description of the *Solemya* symbiosis have been deleted.

2. Figure 1 and 3, please see comments above.

3. The band sizes resulting from the two enzymes not shown in Figure 3, StuI and NcoI, are now stated in the text (page 7).

4. The stringency conditions of hybridization are now stated under the topic "Southern blot and hyridization analysis, 2nd paragraph.

5. References to the chemoautotrophic potential of *Thiothrix nivea* (p. 10, line 14 and p. 14, line 9) have been deleted.

6. The references have been cut by over 1/3 due to overall reduction of the discussion and the rest of the paper.

7. The rRNA operon copy number discussion has been cut to one paragraph. We have included a statement mentioning the possibility that a large enough duplication could allow duplicate rRNA operons to escape detection (page 7). We thought it beyond the scope of this paper to estimate how different theoretically duplicated regions could be and still escape detection.

8. We have eliminated reference to the clustering of the *S. velum* symbiont with *C. burnetti*, and all discussion pertaining to parallels between the *S. reidi* ultrastructure and *C. burnetti*.

JOURNALS DIVISION
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11 March 1992
Accepted: 03/09/92
Article: JB 1468-91

Dr. Colleen M. Cavanaugh
Department of Organismic and
Evolutionary Biology
The Biological Laboratories
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Title: Phylogenetic Relationships of Chemoautotrophic Bacterial Symbionts of *Solemya velum* Say (Mollusca: Bivalvia) Determined by 16S rRNA Gene Sequence Analysis

The above manuscript has been accepted for publication in the *Journal of Bacteriology* by Dr. James D. Friesen, Research Institute, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8 CANADA (phone 416-598-6577, fax 416-591-5085). It has been scheduled to appear in the Genetics and Molecular Biology section of the May '92 issue (volume 174, issue 10) with the implicit understanding that the signed copyright transfer agreement be returned within 3 days of receipt and that the authors will pay the page charge of $50 per published page. By publishing in the journal, the authors agree that any plasmids, viruses, and living materials such as microbial strains and cell lines newly described in the article are available from a national collection or will be made available in a timely fashion and at reasonable cost to members of the scientific community for noncommercial purposes.

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Sincerely,

Sara C. Joslyn ceb

Sara C. Joslyn
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