SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

RAD: 14-139380- -4 **FECHA**: 2016-03-30 15:26:52
DEP: 2020 DIRECCION DE NUEVAS CREACIONES **EVE:** 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II **FOLIOS**: 7
ACT: 519 TRASLADOINFORME

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
ALVARO JOSE CORREA ORDOÑEZ

Asunto: Radicación: 14-139380- -4
Trámite: 11
Evento: 378
Actuación: 519
Oficio: 3352
Folios: 7

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2012/069364				
Fecha de Presentación Internacional	13/12/2012				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado N°14-139380-00000-0000	27/Junio/2014
Reivindicaciones:	Radicado N°14-139380-00000-0000	27/Junio/2014
Secuencias:	Radicado N°14-139380-00000-0000	27/Junio/2014
Prioridad:	US 61 570 553	14/Diciembre/2011

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capitulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por la solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y

Superintendencia de Industria y Comercio: Cra 13 No. 27-00 pisos 1, 3, 4, 5, 6, 7 y 10 - PBX: (571) 5870000
E-mail: contactenos@sic.gov.co - Bogotá D.C., Colombia.
Señor ciudadano la entidad le ofrece los siguientes canales para hacer seguimiento a su solicitud así:
Página web: www.sic.gov.co
Línea gratuita a nivel nacional: 018000 910165
Centro de atención telefónica en Bogotá: 5920400
Kiosco Informático Centro Comercial Gran Estación en Bogotá
MINCOMERCIO INDUSTRIA Y TURISMO

no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso se estudiarán las reivindicaciones 1 a 11 del capítulo reivindicatorio (Radicado N°. 14-139380-00000-0000).

Título de la solicitud: "ENRIQUECIMIENTO DE FLUIDOS DE YACIMIENTOS DE PETRÓLEO CON SHEWANELLA"

El problema planteado en esta solicitud consiste en una necesidad de proporcionar métodos y cepas de microbios útiles para mejorar la recuperación de petróleo.

Para resolver este problema técnico la solicitud en estudio proporciona un método para enriquecer poblaciones microbianas que son autóctonas en fluidos de yacimientos de petróleo para obtener poblaciones enriquecidas con *Shewanella* y cepas aisladas de *Shewanella* útiles para mejorar la recuperación de petróleo de un yacimiento de petróleo.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C12N 1/20, C12P 7/64, C12R 1/01.

4. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El objeto de las reivindicaciones 1-11 no es claro puesto que menciona un método, pero este no define las características técnicas para que pueda llevarse a cabo como, por ejemplo, condiciones del yacimiento (pH, temperatura, etc) para el enriquecimiento con la cepa *Shewanella* que contribuyan a la obtención del efecto técnico planteado, cuál es la composición del medio de crecimiento, cómo se enriquece la población microbiana, etc. Se sugiere a la solicitante definir cómo se lleva a cabo cada etapa, la cepa, el aceptor de electrones, la sal y la fuente de carbón del método reclamado.

El objeto de las reivindicaciones 1, 3, 7, 11 no es claro puesto que hace referencia a "para obtener una población microbiana enriquecida", "*la población microbiana enriquecida de (b) es mayor que la proporción de células de Shewanella en la muestra ambiental", "obtener una segunda población microbiana enriquecida", "para obtener colonias aisladas", "se identifica al menos una cepa de Shewanella" y "el cultivo de la población microbiana enriquecida mejora la recuperación de petróleo"*, lo cual es considerado un resultado a alcanzar y no define el método. Se sugiere a la solicitante caracterizar el método por la secuencia de etapas técnicas y condiciones.

El objeto de las reivindicaciones 1-3, 5-8 y 11 no es claro puesto que hace referencia a las expresiones "al menos", "entre aproximadamente", "al menos aproximadamente", lo cual es impreciso y no permite distinguir el objeto de la solicitud del estado de la técnica. Se sugiere a la solicitante eliminar dichas expresiones y mencionar los rangos concretos.

5. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 14 de diciembre de 2011, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	US20090260803	"Identification, characterization, and application of *Shewanella putrefaciens* (lh4:18), useful in microbially enhanced oil release"	22/Octubre/2009
D2	US5328689	"Induction of settlement and metamorphosis in *Crassostrea virginica* by melanin-synthesizing bacteria and ammonia, and metabolic products of said bacteria"	12/Julio/1994

Superintendencia de Industria y Comercio: Cra 13 No. 27-00 pisos 1, 3, 4, 5, 6, 7 y 10 - PBX: (571) 5870000
E-mail: contactenos@sic.gov.co - Bogotá D.C., Colombia.
Señor ciudadano la entidad le ofrece los siguientes canales para hacer seguimiento a su solicitud así:
Página web: www.sic.gov.co | Línea gratuita a nivel nacional: 018000 910165
Centro de atención telefónica en Bogotá: 5920400 | Kiosco Informático Centro Comercial Gran Estación en Bogotá
MINCOMERCIO INDUSTRIA Y TURISMO

D3	*Applied and Environmental Microbiology,* 2000. 66 (6); 2491 – 2501.	"Isolation and Characterization of Strains CVO and FWKO B, Two Novel Nitrate-Reducing, Sulfide Oxidizing Bacteria Isolated from Oil Field Brine"	Junio/2000

El documento D1[1] divulga el aislamiento e identificación de cepas de *Shewanella putrefaciens* LH4:18 (ATCC No. PTA-8822) que crecen presencia de petróleo en condiciones desnitrificantes empleadas en la recuperación de petróleo (Reivindicaciones 1-17), además hace referencia al método para enriquecer una población microbiana (Párr. [0004], [0005], [0010], [0011]).

El documento D2[2] divulga el aislamiento e identificación de cepas de *Shewanella putrefaciens* en medios con distintas concentraciones de sal (Col. 4, líneas. 46 - 66).

El documento D3[3] divulga cepas de bacterias CVO y FWKO B aisladas de las aguas de producción de un campo de petróleo de Saskatchewan, Canadá. Las cepas FWKO B se identificaron y caracterizaron como nuevas cepas de bacterias de azufre quimiolitotrofas pertenecientes al género *Arcobacter spp*. (Resumen).

6. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en: *"Un método para enriquecer una población microbiana; el método comprende: a) proporcionar una muestra ambiental de un fluido de un yacimiento de petróleo que comprende una población microbiana autóctona; y b) cultivar esa muestra en un medio que comprende sal, al menos una fuente de carbono y un aceptor de electrones seleccionado de aceptor de electrones orgánico y aceptor de electrones de ión metálico para obtener una población microbiana enriquecida; caracterizado porque la proporción de células de Shewanella en la población microbiana enriquecida de (b) es mayor que la proporción de células de Shewanella en la muestra ambiental de (a)"* (Pág. 54 del radicado Nº. 14-139380-00000-00000).

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1
Método para enriquecer una población microbiana que comprende:	Método para enriquecer una población microbiana que comprende: (Párr. [0004])
proporcionar una muestra ambiental de un fluido de un yacimiento de petróleo que comprende una población microbiana autóctona	proporcionar una muestra ambiental de un fluido de un yacimiento de petróleo que comprende una población microbiana autóctona (Párr. [0004]-[0005] y [0028]).
cultivar esa muestra en un medio que comprende sal, al menos una fuente de carbono y un aceptor de electrones seleccionado de aceptor de electrones orgánico y aceptor de electrones de ión metálico; células de *Shewanella*	Cultivos de *Shewanella putrefaciens LH4:18 (ATCC No. PTA-8822)* en un medio que comprende sal (Tabla. 2), una fuente de carbono como lactato (Resumen, párr. [0023]-[0071]) y un aceptor de electrones como nitrato (0048) y aceptor de ion metálico (Párr. [0005]).

Las características esenciales del método para enriquecer una población microbiana de la reivindicación 1, han sido divulgadas previamente en el documento D1, el cual da a conocer un método para enriquecer una población microbiana que comprende proporcionar una muestra ambiental de un fluido de un yacimiento de petróleo que comprende una población microbiana autóctona (Párr. [0004] - [0005] y [0028]) y cultivos de *Shewanella putrefaciens* LH4:18 (ATCC No. PTA-8822) en un medio que comprende

1 http://worldwide.espacenet.com/publicationDetails/biblio?CC=US&NR=2009260803A1&KC=A1&FT=D
2 http://worldwide.espacenet.com/publicationDetails/biblio?CC=US&NR=5328689A&KC=A&FT=D
3 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC110567/pdf/am002491.pdf

Superintendencia de Industria y Comercio: Cra 13 No. 27-00 pisos 1, 3, 4, 5, 6, 7 y 10 - PBX: (571) 5870000
E-mail: contactenos@sic.gov.co - Bogotá D.C., Colombia.
Señor ciudadano la entidad le ofrece los siguientes canales para hacer seguimiento a su solicitud así:
Página web: www.sic.gov.co
Línea gratuita a nivel nacional: 018000 910165
Centro de atención telefónica en Bogotá: 5920400
Kiosco Informático Centro Comercial Gran Estación en Bogotá
MINCOMERCIO INDUSTRIA Y TURISMO

sal (Tabla. 2), una fuente de carbono como lactato (Resumen, párr. [0023] - [0071]) y un aceptor de electrones como nitrato (0048) y aceptor de ion metálico (Párr. [0005]).

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el documento D1.

La reivindicación dependiente 6 no menciona características adicionales que haga nuevo el método de la reivindicación 1, puesto que el documento D1 menciona cultivos de *Shewanella putrefaciens* LH4:18 (ATCC No. PTA-8822) en un medio que comprende sal (Tabla. 2), una fuente de carbono como lactato (Resumen, párr. [0023]-[0071]), un aceptor de electrones como nitrato (0048) y aceptor de ion metálico como cromo, hierro o manganeso (Párr. [0005]), por lo tanto, la reivindicación tampoco se considerada nueva.

Nivel inventivo (Art 18 D486)

La reivindicación 2 consiste en: *"El método de conformidad con la reivindicación 1, caracterizado además porque el medio de (b) tiene una concentración de sal que esta entre aproximadamente 10 ppt y 55 ppt"* (Pág. 54 del radicado Nº. 14-139380-00000-00000).

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D2
Método para enriquecer una población microbiana que comprende:	Método para enriquecer una población microbiana que comprende: (Párr. [0004])	Cultivos de *Shewanella* y medio óptimo de crecimiento (Col. 4, líneas. 46 - 66)
proporcionar una muestra ambiental de un fluido de un yacimiento de petróleo que comprende una población microbiana autóctona	proporcionar una muestra ambiental de un fluido de un yacimiento de petróleo que comprende una población microbiana autóctona (Párr. [0004]-[0005] y [0028]).	No se menciona
cultivar esa muestra en un medio que comprende sal, al menos una fuente de carbono y un aceptor de electrones seleccionado de aceptor de electrones orgánico y aceptor de electrones de ión metálico; células de *Shewanella*	Cultivos de *Shewanella putrefaciens LH4:18 (ATCC No. PTA-8822)* en un medio que comprende sal (Tabla. 2), una fuente de carbono como lactato (resumen, Párr. [0023]-[0071]) y un aceptor de electrones como nitrato (0048) y aceptor de ion metálico (Párr. [0005]).	Cultivos de *Shewanella* (Col. 5, líneas. 5 -15).
el medio tiene una concentración de sal que está entre aproximadamente 10 ppt y 55 ppt	No se menciona	Crecimiento de *Shewanella* en concentraciones de sal entre 15 ppt y 75 ppt (Col. 4, líneas. 60 - 66).

El documento D1 se considera el estado de la técnica más cercano al método reclamado en la reivindicación 2, porque divulga un procedimiento para enriquecer una población microbiana que comprende proporcionar una muestra ambiental de un fluido de un yacimiento de petróleo que comprende una población microbiana autóctona (Párr. [0004] - [0005] y [0028]) y cultivos de *Shewanella putrefaciens* LH4:18 (ATCC No. PTA-8822) en un medio que comprende sal (Tabla. 2), una fuente de carbono como lactato (Resumen, párr. [0023] - [0071]) y un aceptor de electrones como nitrato (0048) y aceptor de ion metálico (Párr. [0005]).

La diferencia entre la solicitud, definida en la reivindicación 2 y el procedimiento del documento D1 consiste en que la solicitud menciona que la concentración de sal en el medio esta entre aproximadamente 10 ppt y 55 ppt.

Superintendencia de Industria y Comercio: Cra 13 No. 27-00 pisos 1, 3, 4, 5, 6, 7 y 10 - PBX: (571) 5870000
E-mail: contactenos@sic.gov.co - Bogotá D.C., Colombia.
Señor ciudadano la entidad le ofrece los siguientes canales para hacer seguimiento a su solicitud así:
Página web: www.sic.gov.co
Línea gratuita a nivel nacional: 018000 910165
Centro de atención telefónica en Bogotá: 5920400
Kiosco Informático Centro Comercial Gran Estación en Bogotá
MINCOMERCIO INDUSTRIA Y TURISMO

El efecto técnico asociado a la concentración de sal del medio entre aproximadamente 10 ppt y 55 ppt es que favorece el crecimiento de *Shewanella* para la recuperación de petroleo.

Por lo tanto, el Problema Técnico Objetivo que pretende resolver esta invención se puede formular así: cómo modificar el proceso del documento D1 con el fin de incrementar la población microbiana de *Shewanella putrefaciens* en un medio con concentración de sal de entre aproximadamente 10 ppt y 55 ppt para su crecimiento.

Sin embargo, el documento D2 divulga cultivos con cepas de *Shewanella* donde el óptimo crecimiento ocurre en un medio cuya concentración de sal es de 35 ppt y un rango de concentración entre 15-75 ppt (Col. 4, líneas. 46 - 66).

En consecuencia, la persona del oficio normalmente versada en la materia, modificaría el método para enriquecer una población microbiana que comprende las etapas de proporcionar una muestra ambiental y un cultivo de la muestra con cepas de *Shewanella putrefaciens* LH4:18 (ATCC No. PTA-8822) en un medio que comprende sal, una fuente de carbono como lactato y un aceptor de electrones como nitrato y aceptor de ion metálico como lo enseña el documento D1, adecuando la concentración de sal en el medio entre 15 – 75 ppt de acuerdo a lo sugerido en el documento D2, con el fin de aumentar la recuperación de petróleo por la acción de microorganismos, para llegar así al objeto de la reivindicación 2 de manera obvia.

En consecuencia, la reivindicación 2 de acuerdo con lo divulgado en los documentos D1 y D2, carece de nivel inventivo.

Las reivindicaciones dependientes 3, 5, 7, 8 y 11 no mencionan características técnicas adicionales por lo tanto tampoco tienen nivel inventivo.

La reivindicación 4 consiste en: *"El método de conformidad con la reivindicación 1, caracterizado además porque el aceptor de electrones es un aceptor de electrones orgánico y caracterizado además porque las células de Arcobacter están presentes en la población microbiana enriquecida de (b)"* (Pág. 55 del radicado Nº. 14-139380-00000-00000).

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	**D1**	**D3**
Método para enriquecer una población microbiana que comprende:	Método para enriquecer una población microbiana que comprende: (Párr. [0004])	Medio para enriquecer una población microbiana (Pág. 2492, col. Izq., líneas. 55-69)
proporcionar una muestra ambiental de un fluido de un yacimiento de petróleo que comprende una población microbiana autóctona	proporcionar una muestra ambiental de un fluido de un yacimiento de petróleo que comprende una población microbiana autóctona (Párr. [0004]-[0005] y [0028]).	Se proporciona una muestra ambiental proveniente del agua que se va a emplear para bombear un pozo (Pág. 2492, col. izq., Materials and Methods).
cultivar esa muestra en un medio que comprende sal, al menos una fuente de carbono y un aceptor de electrones seleccionado de aceptor de electrones orgánico y aceptor de electrones de ión metálico; células de *Shewanella*	Cultivos de *Shewanella putrefaciens LH4:18 (ATCC No. PTA-8822)* en un medio que comprende sal (Tabla. 2), una fuente de carbono como lactato (resumen, Párr. [0023]-[0071]) y un aceptor de electrones como nitrato (0048) y aceptor de ion metálico como cromo, manganeso o hierro (Párr. [0005]).	Cultivo con nitrato como aceptor de electrones (Pág. 2493, col. 5, líneas, 8-25), pág. 2495, tabla 1), crecimiento con concentraciones de NaCl (resumen), acetato como única fuente de carbono (Pág. 2493, col. der.) y aceptor de electrones (Pág. 2492, col. der.).
el medio tiene células de *Arcobacter*	No se menciona	Cepa FWKO B aislada de *Arcobacter*. (Pág. 2492, col.

Superintendencia de Industria y Comercio: Cra 13 No. 27-00 pisos 1, 3, 4, 5, 6, 7 y 10 - PBX: (571) 5870000
E-mail: contactenos@sic.gov.co - Bogotá D.C., Colombia.
Señor ciudadano la entidad le ofrece los siguientes canales para hacer seguimiento a su solicitud así:
Página web: www.sic.gov.co
Línea gratuita a nivel nacional: 018000 910165
Centro de atención telefónica en Bogotá: 5920400
Kiosco Informático Centro Comercial Gran Estación en Bogotá
MINCOMERCIO INDUSTRIA Y TURISMO

		Izq., líneas, 5, 55-69)

El documento D1 se considera el estado de la técnica más cercano al método reclamado en la reivindicación 4, porque divulga un procedimiento para enriquecer una población microbiana que comprende proporcionar una muestra ambiental de un fluido de un yacimiento de petróleo que comprende una población microbiana autóctona (Párr. [0004] - [0005] y [0028]) y cultivos de *Shewanella putrefaciens* LH4:18 (ATCC No. PTA-8822) en un medio que comprende sal (Tabla. 2), una fuente de carbono como lactato (Resumen, párr. [0023] - [0071]), un aceptor de electrones como nitrato (0048) y aceptor de ion metálico como cromo, manganeso o hierro (Párr. [0005]).

La diferencia entre la solicitud, definida en la reivindicación 4 y el procedimiento del documento D1 consiste en que la solicitud menciona la inclusión de un microorganismo adicional de la cepa *Arcobacter*.

El efecto técnico asociado al incluir cepas de *Arcobacter* es mejorar la recuperación de petróleo de yacimientos de petróleo.

Por lo tanto, el Problema Técnico Objetivo que pretende resolver esta invención se puede formular así: cómo modificar el procedimiento divulgado en el documento D1 para mejorar la recuperación de petróleo.

Sin embargo, el documento D3 enseña el empleo de cepas de *Arcobacter* de clado 1 para mejorar la recuperación de petróleo, donde, se aíslan cepas FWKO B de *Arcobacter* spp. de "salmueras" o aguas subterráneas o de superficie; reservorios de agua que será empleada para bombear los pozos, las cuales realizan la mitigación y control de sulfuros (Pág. 2491). Las cepas de *Arcobacter* se pueden mantener en un medio de crecimiento con nitrato como aceptor de electrones (Págs. 2492 y 2493, Tabla. 1) y acetato como única fuente de carbono (Pág. 2493).

En consecuencia, la persona del oficio normalmente versada en la materia, modificaría el procedimiento divulgado en el documento D1, incluyendo dentro del mismo cepas de *Arcobacter* las cuales realizan la mitigación y control de sulfuros según se sugiere en el documento D3, con el fin de aumentar la recuperación de petróleo por la acción de microorganismos, para llegar así al objeto de la reivindicación 4 de la solicitud. Por lo cual se considera obvia.

Por lo tanto, la reivindicación 4 carece de nivel inventivo, según lo divulgado en los documentos D1 y D3.

Por su parte, las reivindicaciones dependientes 9 y 10 no mencionan características adicionales que haga inventivo el método, por lo tanto, carece de nivel inventivo, según lo divulgado en los documentos D1 y D3.

7. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	US20090260803	1 y 6	Novedad
D1	US20090260803	2 a 5, 7, 8 a 11	Nivel inventivo
D2	US5328689	3, 5, 7, 8 y 11	Nivel inventivo
D3	*Applied and Environmental Microbiology,* 2000. 66 (6); 2491 – 2501.	4, 9 y 10	Nivel inventivo

Superintendencia de Industria y Comercio: Cra 13 No. 27-00 pisos 1, 3, 4, 5, 6, 7 y 10 - PBX: (571) 5870000
E-mail: contactenos@sic.gov.co - Bogotá D.C., Colombia.
Señor ciudadano la entidad le ofrece los siguientes canales para hacer seguimiento a su solicitud así:
Página web: www.sic.gov.co
Línea gratuita a nivel nacional: 018000 910165
Centro de atención telefónica en Bogotá: 5920400
Kiosco Informático Centro Comercial Gran Estación en Bogotá
MINCOMERCIO INDUSTRIA Y TURISMO

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Si como respuesta a este requerimiento el solicitante llegara a modificar: i) el capítulo reivindicatorio, ii) la descripción de la invención a proteger, iii) o presente un nuevo capítulo reivindicatorio, no deberá pagar la tasa de modificación voluntaria pero sí la correspondiente a la tasa de examen de patentabilidad para que se estudie la materia modificada. En todo caso, el número de requerimientos por no patentabilidad estará supeditado a que el término del estudio de patentabilidad no sea superior a 18 meses, contados desde la publicación de la solicitud de patente de invención. Adicionalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones deberá pagar la tasa por reivindicación adicional.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.

JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2016-04-05

Elaboró: Juanita Andrea Carvajal
Revisó: Dayron Alexander Ramirez Reyes

Superintendencia de Industria y Comercio: Cra 13 No. 27- 00 pisos 1, 3, 4, 5, 6, 7 y 10 - PBX: (571) 5870000
E-mail: contactenos@sic.gov.co - Bogotá D.C., Colombia.
Señor ciudadano la entidad le ofrece los siguientes canales para hacer seguimiento a su solicitud así:
Página web: www.sic.gov.co
Centro de atención telefónica en Bogotá: 5920400
Línea gratuita a nivel nacional: 018000 910165
Kiosco Informático Centro Comercial Gran Estación en Bogotá
MINCOMERCIO INDUSTRIA Y TURISMO

INFORME DE BÙSQUEDA DE PATENTES PARA EL No de Radicación: 14-139380-00000-0000	
CRITERIOS PARA LA BÙSQUEDA (anexar pantallazo o reporte en PDF, junto con los boleanos utilizados)	
Palabras clave utilizadas	*Shewanella*, yacimiento de petróleo, proceso MEOR.
CLASIFICACIONES INTERNACIONALES UTILIZADAS PARA LA BÚSQUEDA	C12N 1/20, C12P 7/64, C12R 1/01

BASES DE DATOS CONSULTADAS.			
Patentscope	ufrgs		
Google/patents	researchgate		
NCBI	europa		
The lens	fbioyf		

INFORMES DE EXÁMENES REALIZADOS EN OTRAS OFICINAS (documentos patentes, literatura no patente etc…)

Oficina	Fecha del examen o de reporte de búsqueda de documentos	Anterioridades encontradas para el examen o reportados en el examen de búsqueda(relacionarlos todos)	Reiv. afectadas	RELEVANCIA (requisito que afecta)	FUERON TENIDOS EN CUENTA PARA EL EXAMEN EN LA OFICINA COLOMBIANA	
					SI	NO
USPTO	Julio/ 2011	US 20090260803	1 y 11	Nivel Inventivo	X	

DOCUMENTOS ADICIONALES ENCONTRADOS POR EL EXAMINADOR DE ACUERDO A LOS CRITERIOS DE BÙSQUEDA.

Isolation and Characterization of Strains CVO and FWKO B, Two Novel Nitrate-Reducing, Sulfide-Oxidizing Bacteria Isolated from Oil Field Brine.

BÚSQUEDA DE SECUENCIAS
Número de depósito / patente y N° de secuencia
% de identidad

SEQ ID NO (de la solicitud)	ClustalW	The Lens	EMBL-EBI	DDJB	Genome Quest
N.A	-	-	-	-	-

Identification, characterization, and application of ...

www.google.com.co/patents/US7776795 - Traducir esta página
Otorgada - Presentada el 18 Abr. 2008 - Emitido el 17 Ago. 2010 - Sharon Jo Keeler - E.I. Du Pont De Nemours And Company
Shewanella putrefaciens strain LH4:18 can be used alone or in concert ... Microbial Enhanced Oil Recovery (**MEOR**) is a methodology for increasing oil ... in a method of bioremediation of **petroleum** contaminants aerobically.
Descripción general - Información relacionada - Debatir

Application of anaerobic denitrifying bacteria utilizing ...

image not available

www.google.com.co/.../WO2009029500... - Traducir esta página
Solicitud - Presentada el 22 Ago. 2008 - Publicada el 5 Mar 2009 - Linda L Hnatow - Du Pont
Application of anaerobic denitrifying bacteria utilizing **petroleum** components ... miotherma, **Shewanella** putrefaciens, Comamonas terrigena, and Microbulbifer ... Microbial Enhanced Oil Recovery (**MEOR**) is a methodology for ...
Descripción general - Información relacionada - Debatir

Shewanella enrichment from oil reservoir fluids

image not available

www.google.com.co/.../US20130153209 - Traducir esta página
Solicitud - Presentada el 13 Dic. 2012 - Publicada el 20 Jun. 2013 - Edwin Hendrickson - Edwin Hendrickson
One approach for Microbial Enhanced Oil Recovery (**MEOR**) is to .... "**Petroleum**" or "oil" is a naturally occurring, flammable liquid found in rock ...
Descripción general - Información relacionada - Debatir

Identification, characterization, and application of ...

image not available

www.google.com.co/.../WO2009129426... - Traducir esta página
Solicitud - Presentada el 17 Abr. 2009 - Publicada el 22 Oct. 2009 - Sharon Jo Keeler - E. I. Du Pont De Nemours And Company
Shewanella putrefaciens strain LH4:18 can be used alone or in concert with ... Heavy hydrocarbons in the form of **petroleum** deposits and oil reservoirs are ... Microbial Enhanced Oil Recovery (**MEOR**) is a methodology for ...
Descripción general - Información relacionada - Debatir

Shewanella enrichment from oil reservoir fluids

image not available

www.google.com.co/.../CA2857045A1?c... - Traducir esta página
Solicitud - Presentada el 13 Dic. 2012 - Publicada el 20 Jun. 2013 - Edwin Hendrickson - E.I. Du Pont De Nemours And Company
wherein the proportion of **Shewanella** cells in the enriched microbial population of ... One approach for Microbial Enhanced Oil Recovery (**MEOR**) is to .... "**Petroleum**" or "oil" is a naturally occurring, flammable liquid found in ...
Descripción general - Información relacionada - Debatir

Application of anaerobic denitrifying bacteria utilizing ...

image not available

www.google.com.co/.../EP2179063A1?cl... - Traducir esta página
Solicitud - Presentada el 22 Ago. 2008 - Publicada el 28 Abr. 2010 - Linda L. Hnatow - E. I. du Pont de Nemours and Company
Application of anaerobic denitrifying bacteria utilizing **petroleum** components as sole ... **Shewanella** putrefaciens (ATCC#51753), Thauera aromatica S100 ... Microbial Enhanced Oil Recovery (**MEOR**) is a methodology for ...
Descripción general - Información relacionada - Debatir

Shewanella enrichment from oil reservoir fluids

image not available

www.google.com.co/.../WO2013090495... - Traducir esta página
Solicitud - Presentada el 13 Dic. 2012 - Publicada el 20 Jun. 2013 - Edwin Hendrickson - E.I. Du Pont De Nemours And Company
wherein the proportion of **Shewanella** cells in the enriched microbial population ... One approach for Microbial Enhanced Oil Recovery (**MEOR**) is to .... or its components such as **petroleum** hydrocarbons or polycyclic aromatic ...
Descripción general - Información relacionada - Debatir

Identification, characterization, and application of ...

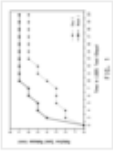
www.google.com.co/.../EP2271740B1?cl... - Traducir esta página
Otorgada - Presentada el 17 Abr. 2009 - Emitido el 22 Mayo 2013 - Sharon Jo Keeler - E. I. du Pont de Nemours and Company
Heavy hydrocarbons in the form of **petroleum** deposits and oil ... Methods for identifying microorganisms useful in **MEOR** processes have been described. ... One strain, designated **Shewanella** putrefaciens LH4:18, was ...
Descripción general - Información relacionada - Debatir

Altering the interface of hydrocarbon-coated surfaces

image not available

www.google.com.co/.../EP2432847A1?cl... - Traducir esta página
Solicitud - Presentada el 21 Mayo 2010 - Publicada el 28 Mar 2012 - Eric R. Choban - E. I. du Pont de Nemours and Company
Shewanella sp of (a); and c) injecting said oil reservoir with a ... Hydrocarbons in the form of **petroleum** deposits and oil reservoirs are distributed worldwide. ... Microbial Enhanced Oil Recovery (**MEOR**) is a methodology for ...
Descripción general - Información relacionada - Debatir

Altering the interface of hydrocarbon-coated surfaces



www.google.com.co/patents/US8658412 - Traducir esta página
Otorgada - Presentada el 21 Mayo 2010 - Emitido el 25 Feb. 2014 - Eric R. Choban - E I Du Pont De Nemours And Company
a) an isolated **Shewanella** sp. having a 16S rDNA that has at least 99% ... Hydrocarbons in the form of **petroleum** deposits and oil reservoirs are distributed worldwide. ... Microbial Enhanced Oil Recovery (**MEOR**) is a methodology for .... Depicts oil release and MPNs of strain **LH4**:**18** in the presence of ...
Descripción general - Información relacionada - Debatir

Altering the interface of hydrocarbon-coated surfaces

image not available

www.google.com.co/.../EP2432847A1?cl... - Traducir esta página
Solicitud - Presentada el 21 Mayo 2010 - Publicada el 28 Mar 2012 - Eric R. Choban - E. I. du Pont de Nemours and Company
The method of claim 1 wherein the **Shewanella** sp. is **LH4**:**18** .... Hydrocarbons in the form of **petroleum** deposits and oil reservoirs are distributed worldwide. ... Microbial Enhanced Oil Recovery (**MEOR**) is a methodology for ...
Descripción general - Información relacionada - Debatir

Altering the interface of hydrocarbon-coated surfaces

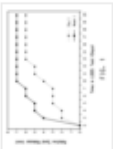
www.google.com.co/patents/US9200191 - Traducir esta página
Otorgada - Presentada el 19 Nov. 2013 - Emitido el 1 Dic. 2015 - Edwin R Hendrickson - E I Du Pont De Nemours And Company
wherein the **Shewanella** sp. comprise a 16S rDNA having at least 99% .... Hydrocarbons in the form of **petroleum** deposits and oil reservoirs are ... Microbial Enhanced Oil Recovery (**MEOR**) is a methodology for .... 1 shows the relative sand release by strain **LH4**:**18** cultures over a period of three weeks.
Descripción general - Información relacionada - Debatir

Shewanella enrichment from oil reservoir fluids

image not available

www.google.com.co/.../WO2013090495... - Traducir esta página
Solicitud - Presentada el 13 Dic. 2012 - Publicada el 20 Jun. 2013 - Edwin Hendrickson - E.I. Du Pont De Nemours And Company
wherein the proportion of **Shewanella** cells in the enriched microbial population of (b) is ... One approach for Microbial Enhanced Oil Recovery (**MEOR**) is to stimulate growth of .... "**Petroleum**" or "oil" is a naturally occurring .... by reference, is isolation of **Shewanella** putrefaciens strain **LH4**:**18** (ATCC No.
Descripción general - Información relacionada - Debatir

Altering the interface of hydrocarbon-coated surfaces



www.google.com.co/.../EP2796527A1?cl... - Traducir esta página
Solicitud - Presentada el 21 Mayo 2010 - Publicada el 29 Oct. 2014 - Abigail K. Luckring - E. I. du Pont de Nemours and Company
Hydrocarbons in the form of **petroleum** deposits and oil reservoirs are distributed ... Microbial Enhanced Oil Recovery (**MEOR**) is a methodology for .... Depicts oil release over time with **LH4**:**18** and other **Shewanella** species

MEOR shewanella petroleum

Todos | Imágenes | Noticias | Videos | Maps | Más | Herramientas d

Cerca de 125,000 resultados (0.54 segundos)

Patent US7776795 - Identification, characterization, and ...
www.google.com/patents/US7776795 - Traducir esta página
17 ago. 2010 - **Shewanella** putrefaciens strain LH4:18 can be used alone or in concert with ... Microbial Enhanced **Oil** Recovery (**MEOR**) is a methodology for ...

Patent WO2013090495A1 - Shewanella enrichment from oil ...
www.google.co.ve/patents/WO2013090495A1?cl... ▾ Traducir esta página
20 jun. 2013 - wherein the proportion of **Shewanella** cells in the enriched microbial ... One approach for Microbial Enhanced **Oil** Recovery (**MEOR**) is to ...
Visitaste esta página 2 veces. Última visita: 18/02/16

[PDF] Differences in microbial community composition between ...
www.biogeosciences.net/12/.../bg-12-3403-2015.pd... ▾ Traducir esta página
por PK Gao - 2015 - Mencionado por 2 - Artículos relacionados
5 jun. 2015 - **oil** recovery (**MEOR**) has been applied to the **petroleum** in- dustry and is currently ..... Oleibacter, Marinobacter, and **Shewanella** were dominant.

[PDF] Microbial Enhanced Oil Recovery (MEOR) - Chemical ...
www.che.ncsu.edu/.../**petroleum**/EOR-Microbial.pdf ▾ Traducir esta página
por I Lazar - 2007 - Mencionado por 136 - Artículos relacionados
Abstract: Microbial enhanced **oil** recovery (**MEOR**) represents the use of microor- ... the **petroleum** crises in 1973, the EOR methods became less profitable.
Falta(n): ~~shewanella~~

Microbial enhanced oil recovery - Wikipedia, the free ...
https://en.wikipedia.org/.../Microbial_enhanced_**oil**_... ▾ Traducir esta página
Pasar a **MEOR** strategies - [edit]. Changing **oil** reservoir ecophysiology to favour **MEOR** can be achieved by complementing different strategies.
Falta(n): ~~shewanella~~

Issues in Fossil Fuel Energy Technologies: 2013 Edition
https://books.google.com.co/books?isbn... - Traducir esta página
2013 - Technology & Engineering
oil. recovery. By a News Reporter-Staff News Editor at Energy Weekly News ... and bioclogging in water-wet micromodels, using **Shewanella** oneidensis (MR-1) ... When analyzing residual **oil** blob morphology, **MEOR** resulted in **oil** blob size ...

PDF(666K) - Wiley Online Library
onlinelibrary.wiley.com/doi/10.1002/mbo3.../pdf ▾ Traducir esta página
por P Gao - 2015 - Mencionado por 6 - Artículos relacionados
7 ene. 2015 - zia, Ochrobactrum, Hyphomonas, Acinetobacter, and **Shewanella** were dominant, ... microbial enhanced **oil** recovery (**MEOR**) are currently.

Microbial diversity and abundance in the Xinjiang Luliang ...
www.ncbi.nlm.nih.gov/pubmed/25641701 - Traducir esta página
por P Gao - 2015 - Mencionado por 6 - Artículos relacionados
2 feb. 2015 - ... enhanced **oil** recovery (**MEOR**) and their abundance in the Xinjiang ... Ochrobactrum, Hyphomonas, Acinetobacter, and **Shewanella** were ...

Analysis of bacterial diversity in two oil blocks from two low ...
www.ncbi.nlm.nih.gov › ... › PubMed Central (PMC) - Traducir esta página
por M Xiao - 2016 - Artículos relacionados
20 ene. 2016 - Microbial enhanced **oil** recovery (**MEOR**) has been proposed as a .... Arcobacter, **Shewanella** and Thalassospira were plentiful in Block Liu, ...

Using Microbes to Vastly Increase Recovery from Old Oil Wells
oilprice.com › Energy › Crude Oil ▾ Traducir esta página
6 ago. 2012 - The best source of **oil** would be to increase **oil** recovery from the existing ... is developed from technology called "microbial enhanced **oil** recovery" (**MEOR**), ... The OSU team used **Shewanella** oneidensis (MR-1) that causes ...

Biodegradación de aceites usados - Monografias.com
www.monografias.com › Ingenieria
Las bases lubricantes sintéticas son fabricadas por **procesos** especiales (distintos ... ahorro de energía, mantenimiento con **menor** frecuencia, **menor** uso de aditivos ..... de la unidad de vacío, formaran después de la redestilación el "spindle **oil**". ..... Las bacterias Acidithiobacillus [21] y **Shewanella** oneidensis [22] tienen la ...

[PDF] Descargar fichero - 39 congreso sebbm 2016
www.sebbm.com/.../libro_resumenes_congreso_SEBBM_Granada_2014...
supervivencia celular, mientras que su déficit promueve **procesos** de inflamación que ..... **petroleum**-based plastics. From an industrial ..... diferenciarlas de aquellas con **menor** probabilidad de desarrollar metástasis. Con este ..... efecto del PB **Shewanella** putrefaciens Pdp11 en el proteoma hepático del lenguado ...

[PDF] Cocoa enriched-diet a... - digital-csic Digital CSIC - Conse...
digital.csic.es/bitstream/10261/.../Cocoa%20enriched-diet%20attenuates.pdf
por E Fernández-Millán - 2014
supervivencia celular, mientras que su déficit promueve **procesos** de inflamación que ..... **petroleum**-based plastics. From an industrial ..... diferenciarlas de aquellas con **menor** probabilidad de desarrollar metástasis. Con este ..... efecto del PB **Shewanella** putrefaciens Pdp11 en el proteoma hepático del lenguado ...

[PDF] Caracterización de los exopolisacáridos ... - Digibug
digibug.ugr.es/bitstream/10481/973/1/16115946.pdf
por JA Mata Gómez - 2006 - Mencionado por 1 - Artículos relacionados
Se ha estudiado en Halomonas elongata un **proceso** de extracción de ectoína conocido como ..... débilmente fermentadoras como **Shewanella** y Ferrimonas, y aerobios estrictos, ... es normalmente **menor** del 1% de la fuente de carbono presente en el medio (De ..... Biotechnology of **petroleum** pollulant biodegradation.

[PDF] NOMBRE Y CLAVE DEL PROYECTO
repositorio.imta.mx:8080/cenca-repositorio/bitstream/.../1/TC-1404.1.pdf
por P Mijaylova Nacheva - 2014 - Artículos relacionados
Los **procesos** de oxidación avanzada son capaces de remover compuestos ..... degradación biológica y su remoción puede ser **menor** al 20%. ..... bacterias como **Shewanella**, mientras que en este estudio solo se produjó una densidad ..... of organic compounds in wáter and soil contaminated with crude **petroleum**.

[PDF] BioTecnología, Año 2009, Vol. 13 No. 3 - Sociedad ...
www.smbb.com.mx/revista/Revista_2009_3/Revista_2009_3.pdf
Los nuevos esquemas de reacción, separación y control en **procesos** biotecnológicos. 3. ..... ideal para la gasolina pues posee un **menor** contenido energético que la gasolina y es ..... waste waters; sustained high growth rates; elevated **oil** productivity; and acceptable fatty acid ..... Las bacterias del género **Shewanella** son.

[PDF] Biocombustibles
www.andaluciabioregion.es/download_file.cfm?id=332&type=2...
16 jun. 2011 - en mayor o **menor** grado, son contaminantes. Desde mediados del ... Tabla 2: **Procesos** de obtención de biocombustibles (Fuente: ..... Continuous production of biodiesel from waste cooking **oil** using green engineering ..... The most used microorganisms in the MFCs belong to **Shewanella**,. Proteobactor ...

[PDF] memorias/proceedings - UNAM
cibernetica.ccadet.unam.mx/.../VII%20Mini-S%20Procs-Mems%2012-1...
12 sep. 2012 - SUE-103 **Procesos** para la biorremediación de suelos y sedimentos ..... Remediation of water contaminated with **petroleum** with Pennisetum clandestinum as ..... elementos ésta es mucho **menor**, se observa que la concentración ..... **Shewanella** Species and Their Role in Extracellular Electron Transfer.

[PDF] View/Open - Instituto Politécnico Nacional
tesis.ipn.mx/.../2004%20JUANA%20MIREYA%20MENDOZA%20VERA....
por M VERA - 2015 - Artículos relacionados
Proceso de aceleración de la tasa de degradación natural de hidrocarburos por .... Psicrofilo: Organismo con un óptimo crecimiento a temperatura **menor** de 20ºc. R ..... En materia de producción de petróleo crudo, durante el año pasado **Oil** and Gas Journal clasificó a ..... Growth of **Geobacter** sulfurreducens with acetate in.

GEOMINAS52 by Revista GEOMINAS - issuu
issuu.com/geominas/docs/geominas52
3 mar. 2011 - Según datos de UNICEF 4.200 niños **menores** de 5 años mueren diariamente ..... considerado uno de los **procesos** de mayor relevancia en la pérdida ..... cultivos de la bacteria **Shewanella** oneidensis MR-1, usando lactato como .... and Archaea in deep subsurface **petroleum** reservoirs of western Siberia.

… conditions to modify the properties of heavy crude **oil** in order to enhance the recovery of the crude **oil** from its underground **reservoir**; production of biosurfactants …
SJ Keeler, ER Hendrickson, LL Hnatow… - US Patent …, 2010 - Google Patents
... Column reactors were used to develop enrichment cultures from industrial and environmental samples to select for a diversity of organisms that would grow on **oil** for use in **MEOR**. **...** Because of the ability of **Shewanella** putrefaciens **LH4**:**18** to release hydrocarbons **...**
Citado por 6 Artículos relacionados Las 6 versiones Citar Guardar

Identification, characterization, and application of Pseudomonas stutzeri (LH4: 15), useful in microbially enhanced **oil** release
SJ Keeler, RD Fallon, ER Hendrickson… - US Patent …, 2013 - Google Patents
... were used to develop enrichment cultures from industrial and environmental samples to select for a diversity of organisms that would grow on **oil** for use in **MEOR**. **...** In some embodiments, the additional agent is **Shewanella** putrefaciens strain **LH4**:**18**, which is **...**
Citado por 2 Artículos relacionados Las 6 versiones Citar Guardar

Microbial EOR: Critical Aspects Learned From the Lab
SC Jackson, AW Alsop, ER Choban… - SPE Improved **Oil** …, 2010 - onepetro.org
... S. J Keeler, ER Hendrickson, LL Hnatow, SC Jackson, Identification, Characterization and Application of **Shewanella** putrefaciens (**LH4**:**18**), Useful in **...** al,: A Novel and Successful **MEOR** Pilot Project in a Strong Water-Drive **Reservoir** Vizcacheras Field, Argentina **...**
Citado por 3 Artículos relacionados Las 2 versiones Citar Guardar

Shewanella enrichment from **oil reservoir fluids**
E Hendrickson, A Luckring - US Patent App. 13/713,064, 2012 - Google Patents
... Use of **Shewanella** and Arcobacter Cells for **MEOR ...** 7,776,795, which is incorporated herein by reference, is isolation of **Shewanella** putrefaciens strain **LH4**:**18** (ATCC No. **...** The nucleotide sequence of the 16S rDNA used to identify the **Shewanella** homology cluster was SEQ ID **...**
Artículos relacionados Las 2 versiones Citar Guardar

Altering the interface of **hydrocarbon**-coated surfaces
ER Choban, RD Fallon, ER Hendrickson… - US Patent …, 2014 - Google Patents
... Certain microorganisms have been described as having properties that may benefit **MEOR** processes. **...** PTA-8822, Dec. 4, 2007. **LH4**:**18**, **...** Treatment may also involve pumping water with cell-free medium produced by conditioning with **Shewanella** into a treatment zone. **...**
Artículos relacionados Las 4 versiones Citar Guardar

Prevention of contamination of feed **reservoirs** & feed lines in bioreactor systems
RD Fallon - US Patent App. 13/297,514, 2011 - Google Patents
... An example of a **MEOR** process is described in commonly owned and co-pending US Patent Application Publication #2011/0030956, which is herein incorporated by reference. **...** In another embodiment **Shewanella** putrefaciens **LH4**:**18** (ATCC PTA-8822) cells can **...**
Artículos relacionados Las 2 versiones Citar Guardar

inoculation of **reservoirs**; anaerobic; bioremediation; denitrification
ER Hendrickson, RE Jackson, SJ Keeler… - US Patent …, 2010 - Google Patents
... MEOR research and development is an ongoing effort directed at discovering techniques to **...** of metabolizing hydrocarbons or other organic components of crude **petroleum** as a **...** cell surface hydrophobicity and (bio)surfactant production in many **hydrocarbon**-associated microbes **...**
Citado por 8 Artículos relacionados Las 4 versiones Citar Guardar

Prevention of contamination of nutrient feed **reservoirs** & feed lines in bioreactor
SC Jackson - US Patent App. 13/297,634, 2011 - Google Patents
... The present bioreactor system and method may be used to grow cells for use in **MEOR** processes, an example of which is described in commonly owned and co-pending US Patent Application **...** In another embodiment **Shewanella** putrefaciens **LH4**:**18** (ATCC PTA **...**
Artículos relacionados Las 2 versiones Citar Guardar

Method and system for predicting the effect of microbes injected into an **oil**-bearing **reservoir**
JG Beattie, AS Davies, DA Puckett… - US Patent …, 2014 - Google Patents
... are designed to allow efficient propagation through the pore structure of **hydrocarbon reservoir** matrix rock **...** be selected from one or more microbes from the following genera: **Shewanella**, Pseudomonas, Bacillus **...** In order to predict the effects of an **MEOR** flood on the amount of **oil ...**
Citado por 1 Artículos relacionados Las 4 versiones Citar Guardar

Altering the interface of **hydrocarbon**-coated surfaces
ER Hendrickson, AK Luckring, MP Perry - US Patent 9,029,123, 2015 - Google Patents
... Certain microorganisms have been described as having properties that may benefit **MEOR** processes. **... LH4**:**18**, **...** or biomolecules produced by **Shewanella** sp., and **hydrocarbon** coated surfaces produces alterations in the wettability properties of the **hydrocarbon** coated surface **...**
Artículos relacionados Las 4 versiones Citar Guardar

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 2000, p. 2491–2501 Vol. 66, No. 6
0099-2240/00/$04.00+0
Copyright © 2000, American Society for Microbiology. All Rights Reserved.

Isolation and Characterization of Strains CVO and FWKO B, Two Novel Nitrate-Reducing, Sulfide-Oxidizing Bacteria Isolated from Oil Field Brine

DIANE GEVERTZ,[1] ANITA J. TELANG,[2] GERRIT VOORDOUW,[2] AND GARY E. JENNEMAN[3]*

The Agouron Institute, La Jolla, California 92037[1]; Department of Biological Sciences, University of Calgary, Alberta, Canada T2N 1N4[2]; and Phillips Petroleum Company, Bartlesville, Oklahoma 74004[3]

Received 29 November 1999/Accepted 28 March 2000

Bacterial strains CVO and FWKO B were isolated from produced brine at the Coleville oil field in Saskatchewan, Canada. Both strains are obligate chemolithotrophs, with hydrogen, formate, and sulfide serving as the only known energy sources for FWKO B, whereas sulfide and elemental sulfur are the only known electron donors for CVO. Neither strain uses thiosulfate as an energy source. Both strains are microaerophiles (1% O_2). In addition, CVO grows by denitrification of nitrate or nitrite whereas FWKO B reduces nitrate only to nitrite. Elemental sulfur is the sole product of sulfide oxidation by FWKO B, while CVO produces either elemental sulfur or sulfate, depending on the initial concentration of sulfide. Both strains are capable of growth under strictly autotrophic conditions, but CVO uses acetate as well as CO_2 as its sole carbon source. Neither strain reduces sulfate; however, FWKO B reduces sulfur and displays chemolithoautotrophic growth in the presence of elemental sulfur, hydrogen, and CO_2. Both strains grow at temperatures between 5 and 40°C. CVO is capable of growth at NaCl concentrations as high as 7%. The present 16s rRNA analysis suggests that both strains are members of the epsilon subdivision of the division *Proteobacteria*, with CVO most closely related to *Thiomicrospira denitrifcans* and FWKO B most closely related to members of the genus *Arcobacter*. The isolation of these two novel chemolithotrophic sulfur bacteria from oil field brine suggests the presence of a subterranean sulfur cycle driven entirely by hydrogen, carbon dioxide, and nitrate.

During commercial production of petroleum, water is often coproduced with the oil. This produced water may be formation water (i.e., stratal) or any mixture of formation water, ground water, and surface water. Ground water or surface water is often injected into petroleum reservoirs to maintain reservoir pressure and improve the recovery of additional oil (12). Produced water, often referred to as "brine" in this paper, usually contains high concentrations of inorganic dissolved solids, such as sodium, chloride, magnesium, calcium, bicarbonate, and sulfate. However, dissolved solids in oil field brines can become more dilute following many years of injection with less-saline ground water or surface water. Many oil field brines also contain sulfides (H_2S and HS^-) as a result of the activity of sulfate-reducing bacteria (SRB) or other sulfidogenic bacteria. Sulfides are undesirable during oil production operations because of their toxicity, corrosiveness, and ability to form insoluble metal sulfides that can lead to a loss of reservoir permeability (40). Typically, control of SRB involves the use of oxidizing and nonoxidizing biocides, such as chlorine, bromine, aldehydes, amines, and quaternary phosphonium salts (28, 45). However, these chemicals can also be toxic, expensive, and ineffective (28, 62).

An alternative method for the mitigation and control of sulfides in oil field brines involve the addition of nitrate. Nitrates stimulate the activity of indigenous nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB) to lower sulfide concentrations as well as stimulating heterotrophic denitrifiers that can outcompete SRB for energy sources (e.g., organic acids). The use of nitrate to control H_2S odors in sewer and other wastewater streams has been known for many years and continues to be of commercial interest (1, 5, 8, 16, 22, 47). More recently, Jenneman et al. (32) and Jack et al. (27) demonstrated that addition of nitrate to sulfide-laden oil field brines could also be used to remove sulfides in these waters. McInerney et al. (41) added ammonium nitrate to an injector at the Southeast Vasser Vertz Sand Unit in Oklahoma and reported a 40 to 60% reduction in sulfide at three adjacent producing wells that they attributed to the activity of indigenous nitrate reducing bacteria (NRB). Reinsel et al. (48) observed lower concentrations of sulfides in the effluents of sandstone columns inoculated with produced water containing indigenous bacteria from two North Sea oil fields following injection with low concentrations of nitrate or nitrite (0.57 to 0.71 mM). However, they attributed the lower sulfide concentrations to nitrite inhibition of the indigenous SRB and not to oxidation of sulfides by indigenous NR-SOB. Sandbeck and Hitzman (50) claimed that nitrate controls sulfide in oil field brines by allowing the indigenous NRB to outcompete SRB for common electron donors (e.g., organic acids), thereby allowing NRB to exclude SRB (i.e., "biocompetitive exclusion"). Furthermore, they suggested that nitrate or nitrite in the presence of low concentrations of molybdate, an SRB inhibitor, is biocidal to SRB (24).

To further demonstrate the effectiveness of nitrate in mitigating sulfides in oil field brines, a recent field test was performed at the Coleville (CV) oil field in Saskatchewan, Canada (33). Ammonium nitrate (5 mM) and sodium phosphate (0.1 mM) were added continuously to injected brine for 50 days, resulting in complete removal of sulfide at one of two injectors used in the study. Reductions of 50 to 60% in sulfide levels at two adjacent producing wells were reported (33). Using nitrate-amended enrichments of CV brine, the products of this biological sulfide oxidation were determined to be elemental sulfur and dinitrogen gas (31). Telang et al. (61), using reverse

* Corresponding author. Mailing address: Phillips Petroleum Company, Phillips Research Center, 224 GB, Bartlesville, OK 74004. Phone: (918) 661-8797. Fax: (918) 662-2047. E-mail: gejenne@ppco.com.

sample genome probing, demonstrated that sulfide reductions in the oil field brine were accompanied by significant increases in a novel NR-SOB, referred to as strain CVO, while SRB concentrations remained unchanged or decreased slightly. Another novel NR-SOB, strain FWKO B, was also isolated and purified from CV-produced water but was not a dominant member of nitrate-amended CV brine enrichments. A preliminary report on the isolation and identification of strains CVO and FWKO B has been published (30). Voordouw et al. (70) provisionally identified CVO as a *Campylobacter* sp. and determined its presence in CV brine as well as several other western-Canadian oil field brines using reverse sample genome probing and hybridization of PCR-amplified 16S rRNA genes with a specific oligonucleotide probe. This led to the conclusion that NR-SOB may play key roles in the cycling of sulfur in these oil field brines. The purpose of this paper is to further describe and compare the properties of these two novel chemolithotrophic NR-SOB isolated from oil field brine.

MATERIALS AND METHODS

Source and collection of inoculum. Produced water from the CV oil field near Kindersley, Saskatchewan, Canada, was used for enrichments and bacterial isolations. The water was collected near the bottom of the free-water-knockout (FWKO) tank which is used for primary separation of water, oil, and gas from producing wells. Approximately 4,800 m^3 of water per day is produced, separated, and reinjected into 120 injection wells. Water is reinjected into the Bakken sandstone reservoir at a depth of 823 m with a bottomhole temperature of 29°C. The major constituents of the produced water include (all wt/vol) sodium, 0.29%; chloride, 0.41%; bicarbonate, 0.19%; and sulfate, 0.026%. The pH of the produced water at atmospheric pressure was 7.0 to 8.5. Total dissolved sulfide concentrations were between 100 and 200 mg/liter. The produced oil is a viscous asphaltic crude oil with a density of 0.984 g/cm^3 at 15°C.

Produced water was collected in 1-liter sterile glass bottles that had been preincubated in an anaerobic chamber containing 10% H_2, 5% CO_2, and 85% N_2 (Coy Laboratory Products, Inc., Ann Arbor, Mich.). Following collection, samples were shipped to the laboratory within 24 h and stored in the anaerobic chamber at room temperature. The time between sample collection and use varied between 3 days and 3 weeks.

Growth media. CV synthetic brine (CSB) medium, a modified version of DTA medium (see below) used for growth of *Beggiatoa*, contained (all g/liter) NaCl, 7.0; $MgSO_4 \cdot H_2O$, 0.68; $CaCl_2 \cdot H_2O$, 0.24; NH_4Cl, 0.02; KH_2PO_4, 0.027; $NaC_2H_3O_2 \cdot 3H_2O$, 0.68; KNO_3, 1.0; $NaHCO_3$, 1.90; resazurin, 0.0001; and ND trace metals, 50 ml/liter (43). Following adjustment of the medium pH to between 7.0 and 7.5 and autoclaving, cooling, and equilibration of the medium with chamber gas overnight, sulfide (0.5 to 1.0 mM) was added aseptically from a sterile stock solution of 1.0 M $Na_2S \cdot 9H_2O$ and the medium was dispensed into sterile serum bottles or Balch tubes (18 by 150 mm; Bellco Glass, Inc., Vineland, N.J.) preincubated in the chamber overnight. The bottles were sealed with butyl rubber stoppers and aluminum crimp caps.

DTA brine medium was a semisynthetic CV brine that contained (all g/liter) $(NH_4)_2SO_4$, 0.13; KNO_3, 1.0; KH_2PO_4, 0.03; $NaC_2H_3O_2 \cdot 3H_2O$, 0.78; $CaCl_2$, 0.1; resazurin, 0.01; and 50 ml of ND trace metals (43)/liter added to CV oil field brine collected from the FWKO tank. This medium was sterilized by filtering it through a 0.22-μm-pore-size filter inside the anaerobic chamber or was dispensed into oxygen-free screw-cap glass bottles inside the anaerobic chamber and then sterilized by autoclaving (15 min at 121°C).

Enrichment and isolation. Enrichments were prepared by adding 50 ml of produced water to sterile serum bottles preincubated for at least 24 h in the anaerobic chamber. Potassium nitrate (10 mM) and sodium phosphate, monobasic (0.1 mM), were then added from sterile stock solutions. The bottles were sealed and incubated at 30°C. Strain CVO was isolated by plating enrichments onto S-8 medium plates used for cultivation of *Thiobacillus denitrificans* (26). Isolated colonies from these plates were placed into DTA brine medium. Several rounds of endpoint dilution were used to further purify strain CVO. Strain FWKO B was isolated by plating enrichments in DTA brine medium on DTA brine medium plates. Three rounds of dilution to extinction in DTA brine medium were then used to further purify strain FWKO B. Extinction dilution was used to further purify CVO and FWKO B due to their poor growth on agar-based media used for primary isolation. CVO and FWKO B have been deposited in the Agricultural Research Service culture collection under accession numbers NRRL B-21473 and NRRL B-21472, respectively.

Growth and maintenance. Routine growth and maintenance of both isolates were in CSB medium. Stock cultures of CVO and FWKO B were stored as lyophiles at −40°C. Working cultures from the lyophiles were grown in CSB medium soft agar stabs (0.4% agar). From the stabs, the isolates were cultured into serum bottles containing CSB medium.

Growth was detected from an increase in optical density (at 600 nm) or an increase in acridine orange direct counts (AODC). EpiCount membrane filters and stain (Nuclepore Corp., Pleasanton, Calif.) were used, and the EpiCount procedure was followed except that the membranes were rinsed with 3.0 ml of filtered (0.22-μm pore size) distilled water prior to being stained. An increase in optical density of at least 0.1 absorbance unit or at least a 10-fold increase in AODC was considered an indicator of significant cell growth. In addition, growth on elemental sulfur as an electron donor or acceptor was detected as an increase in sulfate or sulfide concentration, respectively, relative to uninoculated controls.

Growth of type cultures. The following strains of sulfide-oxidizing bacteria were purchased from the American Type Culture Collection (ATCC): *Thiobacillus denitrificans* ATCC 23642, *Thiomicrospira denitrificans* ATCC 33889, *Sulfurospirillum deleyianum* ATCC 51133, *Campylobacter* sp. strain DSM 806, and *Arcobacter nitrofigilis* ATCC 33309. All type strains were grown in media as recommended by the ATCC (20).

Microscopic characterization. All cells used in microscopic characterization were grown in CSB medium. The morphologies and dimensions of isolates were determined from photomicrographs using scanning electron microscopy (SEM), phase microscopy, or epifluorescence microscopy. The widths and lengths given represent the averages of measurements of several cells. Motility and the presence of spores were determined using phase microscopy of wet mounts. Gram staining was performed by the Hucker method (14).

For SEM, 1 ml of either CVO or FWKO B cells grown in CSB medium for about 24 h was centrifuged at 10,000 × *g* for 15 min. The pellet was resuspended in 1 ml of 0.1 M sodium phosphate (pH 7.0) containing 2.5% glutaraldehyde. After 30 min, the cells were washed three times with 0.1 M phosphate buffer and then fixed in 0.1% (wt/vol) osmium tetroxide. Following three more washes in 0.1 M phosphate buffer, a small portion of the cell suspension was removed and washed three times with distilled water. This suspension was pipetted onto a glass slide. Twenty minutes later, the slide was immersed in a liquid nitrogen-cooled isopentane bath. The frozen slides were dried overnight in a lyophilizer, sputter coated with Au-Pd, and observed with a scanning electron microscope.

Enzyme tests. Cells of both strains were grown on CSB agar plates containing 1.0 mM sulfide. Colonies were used to determine oxidase activity with oxidase differentiation disks (Difco Laboratories, Detroit, Mich.) and catalase activity by the Tween 80-hydrogen peroxide assay (10).

Anaerobic substrate tests. Sterilized, anaerobically equilibrated CSB medium was used for all substrate utilization tests. A 2.0 to 2.5% (vol/vol) inoculum of a 24- to 48-h-old culture of strain CVO or FWKO B grown in CSB medium with 0.5 mM sulfide was used. Balch tubes and bottles (50 ml) were sealed and incubated in the dark. Tests with elemental sulfur as an electron donor were performed by adding a slurry of elemental sulfur prepared according to the method of Widdel and Pfennig (72) for growth of *Desulfuromonas*. Ascorbic acid (0.1 g/liter) and thioglycollic acid (0.1 g/liter) were added as reducing agents in tests where sulfide was not the electron donor. Following inoculation, the headspaces in some tubes were exchanged and repressurized (172 kPa) with deoxygenated gas using a gassing manifold (4). All tests were run in triplicate, and growth was determined after at least 5 days of incubation.

(i) Nitrogen reduction. Concentrated stock solutions of the following electron donors were prepared and added to CSB medium, minus sulfide and acetate, at a final concentration of 5 mM: acetate, succinate, formate, lactate, pyruvate, malate, fumarate, and propionate.

Tests with hydrogen as the electron donor were performed in CSB medium minus sulfide and acetate. The headspace was evacuated and replaced with H_2-CO_2 (90%:10%).

Tests with sulfide (0.5 mM) as the electron donor were performed in CSB medium equilibrated with chamber gas at atmospheric pressure. Tests with sodium thiosulfate (10 g/liter) or elemental sulfur (0.03 g/liter) as an electron donor were performed in CSB medium without sulfide using a headspace with N_2-CO_2 (90%:10%). The alternate electron acceptors, sodium nitrite (3.0 mM) and nitrous oxide (2%), were substituted for the nitrate in CSB medium in some tests. Nitrous oxide (Matheson, East Rutherford, N.J.) was added with a gas-tight syringe to the headspaces of bottles containing N_2-CO_2 (90%:10%).

(ii) Sulfur reduction. Tests with hydrogen as the electron donor were performed in CSB medium minus nitrate, sulfide, and acetate, with elemental sulfur added as the electron acceptor and a headspace consisting of H_2-CO_2 (90%:10%).

(iii) Fermentation. The following substrates were added to CSB medium minus nitrate, acetate, and sulfide (all 5 mM): acetate, pyruvate, succinate, fumarate, malate, aspartate, lactate, and glucose.

Aerobic substrate tests. Aerobic growth in CSB medium minus acetate and nitrate was tested for the following organic substrates (all 5 mM): acetate, succinate, lactate, fumarate, malate, formate, pyruvate, glucose, and glycerol. Aerobic growth on thiosulfate (5 mM) and elemental sulfur was tested in CSB medium without nitrate, bicarbonate, and sulfide in 250-ml flasks containing 50 ml of medium with shaking on an incubator-shaker at 250 rpm.

Microaerophilic (1% O_2) growth with sulfide (1.0 mM) as an electron donor was tested in CSB medium without nitrate, whereas S^0 and H_2 as the electron donors were tested in CSB medium without acetate and sulfide. The oxygen was added as 0.5 ml of sterile air (21% O_2) to the headspace of each bottle containing 10 ml of N_2-CO_2 (90%:10%). A control for chemical oxidation of sulfide was run in sterile CSB medium without nitrate containing 1% oxygen in the headspace.

Carbon sources. The ability of CVO and FWKO B to use CO_2 as the sole source of carbon while growing on sulfide-nitrate was tested in CSB medium, with or without acetate. The headspace consisted of 90% N_2 and 10% CO_2. The utilization of acetate (10 mM) as the sole carbon source was tested in CSB medium containing 5 mM KH_2PO_4, minus sodium bicarbonate, and in the presence of an N_2 headspace. Disappearance of sulfide and an increase in AODC were used to follow growth.

Growth parameters. CSB medium containing 2 to 3 mM sulfide was used to study the effects of temperature and salinity, and CSB medium with 1.0 mM sulfide was used to study the effect of pH. The buffers (0.1 mM) used in pH experiments were citrate-phosphate (pHs 5.5 and 6.0), phosphate (pH 7.0), and Tris-hydrochloride (pH 8.5) (11). Growth was defined as the complete oxidation of sulfide within 7 days. All tests were run in triplicate. Controls without added cells were run to determine abiotic oxidation of sulfides.

Growth curves for both CVO and FWKO B were determined for cells growing in DTA brine medium. The medium was filter sterilized and dispensed into sterile 50-ml serum bottles. The bottles were inoculated with CVO and FWKO B previously grown in the same medium, and incubated. Specific growth rates were calculated for data (log AODC versus time) collected during early exponential growth. The data represent averages of three replicates.

Transformations of nitrate-nitrogen and sulfide-sulfur. (i) Effect of sulfide concentrations. Strains CVO and FWKO B were grown in CSB medium with 0.5, 1.0, 2.0, or 3.0 mM sulfide. After 48 h of incubation, samples of culture media were analyzed for sulfide, soluble sulfur compounds (SSC), sulfate, sulfite, thiosulfate, nitrate, and nitrite.

(ii) ^{15}N isotope studies. For growth of CVO and FWKO B, CV field brine was amended with 5 mM $K^{15}NO_3$ and 0.1 mM NaH_2PO_4. For the growth of FWKO B, $(NH_4)_2SO_4$ (130 mg/liter), $CaCl_2$ (100 mg/liter), and 50 ml of ND trace metals (43) were also added. The medium was then filter sterilized (0.22-μm pore size) and dispensed as 100-ml aliquots into sterile 120-ml serum bottles. Following inoculation and sealing, the cultures were incubated overnight and analyzed for nitrate, nitrite, sulfate, sulfite, thiosulfate, sulfur, ammonia, N_2O, and N_2. The presence of $^{15}N_2O$ and $^{15}N_2$ was determined by gas chromatography-mass spectrometry analysis of headspace gas.

Analytical procedures. Sulfide was detected either colorimetrically by a modification of the method of Fogo and Popowski (18) or using an Aquaquant hydrogen sulfide test kit (EM Sciences, Gibbstown, N.J.). Nitrate, nitrite, sulfate, thiosulfate, and sulfite were separated by ion chromatography and detected amperometrically. Ammonia was detected using an ion-specific electrode. Total SSC (which includes sulfate, sulfide, and thiosulfate), elemental sulfur, and calcite were qualitatively identified in culture broth by filtering solids onto a 0.22-μm-pore-size membrane filter, washing them with deionized water, drying them at room temperature, and analyzing them by X-ray diffraction spectroscopy. Elemental sulfur was analyzed by the method of Chan and Suzuki (9) using sulfur extraction in petroleum ether and ferric thiocyanate color formation for detection.

Molecular biology reagents. Deoxyoligonucleotides were obtained from University Core DNA Services, The University of Calgary. PCR reagents (including *Taq* polymerase) and [α-^{35}S]dATP were from Amersham-Pharmacia. *Campylobacter* sp. strain DSM 806 genomic DNA was kindly provided by W. Schumacher, Swiss Federal Institute for Environmental Science and Technology, Kastanienbaum, Switzerland.

Construction of a CVO-specific radiolabeled oligonucleotide probe (CLI) and hybridization test. Whole cells (1 ml at 5×10^7/ml) were applied to a nylon membrane by slot blotting. The cells were then lysed, and the membrane was treated according to the method of Braun-Howland et al. (7). The membrane was then probed with the deoxyoligonucleotide CLI (5′-ATATGCTACCGTCA TT) end labeled with [α-^{32}P]ATP or with the general eubacterial probe EUB. Hybridization conditions have been previously described (70).

16S rRNA gene sequence analysis and phylogeny. Nearly complete 16S rRNA gene sequences (positions 49 to 1313; *Escherichia coli* numbering) were obtained for CVO, FWKO B, and *Campylobacter* sp. strain DSM 806, using both manual sequencing with a Promega fmol cycle-sequencing kit and automated sequencing with an ABI PRISM dye terminator cycle-sequencing ready-reaction kit (Big Dyes). Automated sequencing was done on a model 377XL PRISM Sequencer (Applied Biosystems Inc.) at University Core DNA Services. The 1.4-kb PCR fragments used as templates for sequencing were amplified from genomic DNA with primers f8 (44) and r1406 (23) as explained elsewhere (61). The primers used for sequencing were f8, r1406, EUB 338 (3), p75 [ACCGCGGC(G/T)GC TGGC], p76 (complement of p75), and p78 [GTGAAAT(T/C)CGTAGA(G/T) ATC]. Sequences were aligned with the Staden package (56). The consensus sequence derived from this alignment was compared with all sequences in GenBank with a Blast search (2). Nineteen sequences that showed high homology with CVO, FWKO B, or DSM 806 were retrieved from GenBank. A multiple sequence alignment and corresponding dendrogram of these sequences and those of CVO, FWKO B, DSM 806, and *E. coli* was generated with the PileUp program of the Wisconsin Package version 9.1 (Genetics Computer Group [GCG], Madison, Wis.). The file of aligned sequences was also used to generate a phylogenetic tree with the phylogenetic analysis using parsimony (PAUP) software package (60) with 100 bootstrap replicates and using the *E. coli* sequence as an outgroup.

Nucleotide sequence accession numbers. The sequences for CVO, FWKO B, and *Campylobacter* sp. strain DSM 806 have been deposited in GenBank under accession numbers U465062, AF144693, and AF144694, respectively.

RESULTS

Enrichment and isolation. Addition of nitrate and phosphate to produced water from the CV oil field resulted in the anaerobic biological oxidation of sulfide (3 to 4 mM) and the production of elemental sulfur and dinitrogen gas according to the following stoichiometry (31): $5HS^- + 2NO_3^- + 7H^+ \rightarrow 5S^0 + N_2 + 6H_2O$.

Microscopy of bacteria in the brine revealed the presence of nearly 10^7 cells/ml. SRB, denitrifying bacteria, fermentative bacteria, and spore-forming bacteria have all been previously detected in this brine (reference 70 and unpublished results). Lactate-oxidizing SRB compose nearly 1% of the total population, while NR-SOB have been detected at concentrations of 10^4 to 10^6/ml (20, 33). The NR-SOB, strains CVO and FWKO B, were isolated from enrichments of CV brine containing nitrate and phosphate as colonies on agar plates containing thiosulfate (26) and sulfide as energy sources, respectively. Subsequent purification was by dilution to extinction in DTA brine medium.

Characterization of isolates. CVO (Fig. 1A and B) and FWKO B (Fig. 1C and D) are slightly curved rods with a diameter of 0.4 μm and lengths of 0.6 to 0.8 and 1.0 to 2.0 μm, respectively. Both are gram-negative non-spore formers. FWKO B is very motile, while CVO is nonmotile or very weakly motile. Both strains are catalase negative, while only CVO is positive for oxidase activity (Table 1).

Growth rates. Consumption of sulfide (2 mM) in DTA brine medium was roughly proportional to growth for CVO and FWKO B (Fig. 2). Both strains reached a maximum cell density of over 10^8/ml at specific growth rates of 0.53 and 0.46 h^{-1}, respectively.

Anaerobic growth on nitrate, nitrite, and nitrous oxide. CVO grew anaerobically with sulfide (0.5 to 3.0 mM) as an electron donor and either nitrate, nitrite, or nitrous oxide (2%) as an electron acceptor. In contrast, FWKO B only grew with nitrate as an electron acceptor (Table 1). Growth on sulfide (0.5 mM) and nitrous oxide (2%) by strain CVO was slow, as only a fourfold increase in cell numbers and a 59% decrease in sulfide were observed, on average, after 5 days of incubation. At this time, the addition of more nitrous oxide (2%) did not result in any further sulfide oxidation or growth (results not shown). Sterile controls containing sulfide and nitrous oxide indicated no significant chemical oxidation of sulfide during this time. Both strains are capable of strictly autotrophic growth on sulfide and nitrate, whereas CVO presumably utilized acetate as a carbon source in the absence of added bicarbonate or CO_2 (Table 2). FWKO B displayed hydrogenotrophic growth in the presence of nitrate. Growth of CVO on elemental sulfur in the presence of nitrate or nitrite was accompanied by sulfate production and clumping of sulfur crystals. Neither strain displayed an organotrophic mode of growth in the presence of nitrate and various organic substrates tested (Table 1). In addition, neither CVO nor FWKO B grew in thiosulfate-based media designed for the growth of *Thiobacillus denitrificans* (26) and *Thiomicrospira denitrificans* (66).

Anaerobic growth on sulfate and sulfur. FWKO B grew by reduction of elemental sulfur to sulfide using hydrogen as an electron donor and CO_2 as its sole carbon source (Tables 1 and 2). Attempts to growth CVO and FWKO B in a medium

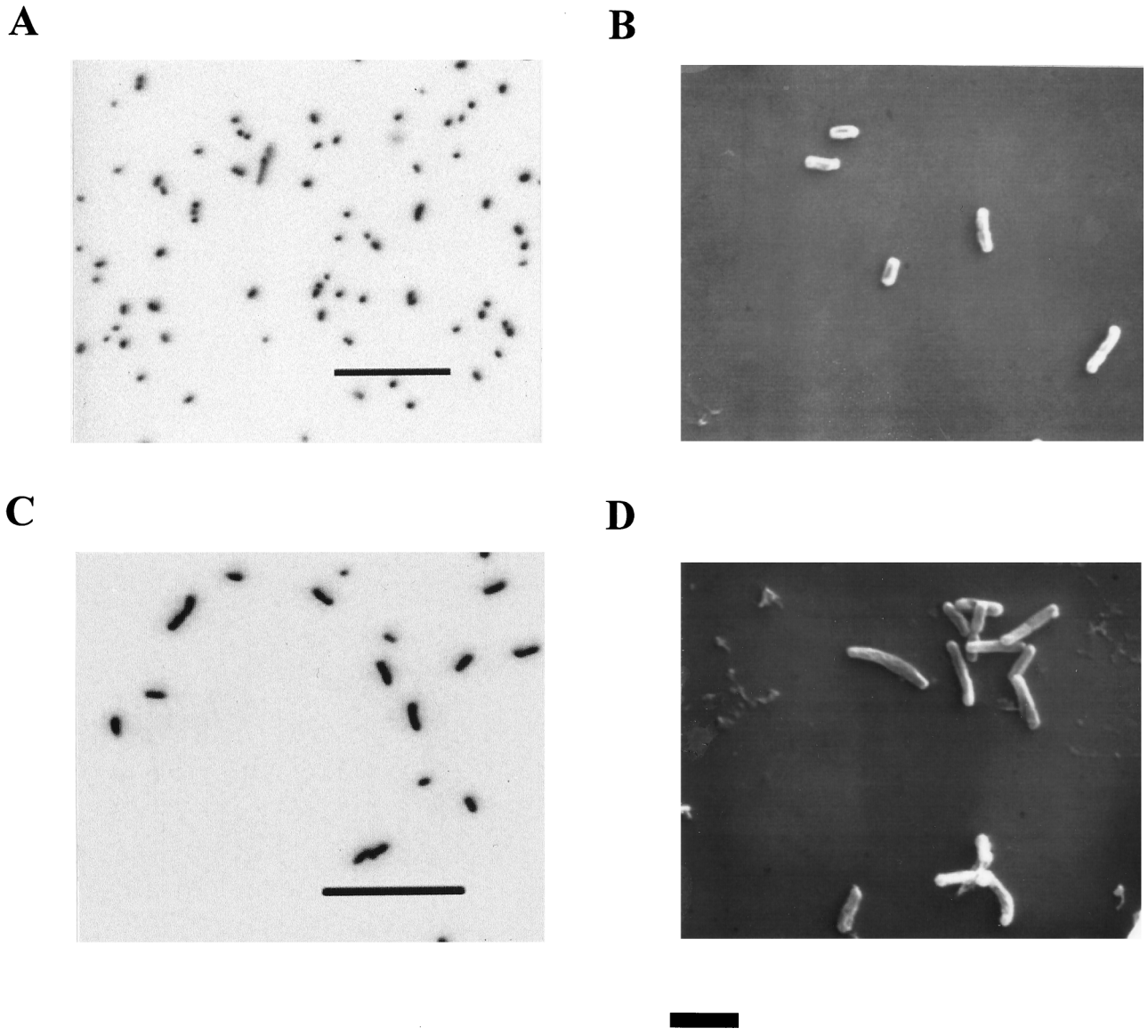


FIG. 1. (A and C) Epifluorescence photomicrographs of strains CVO (A) and FWKO B (C) grown with sulfide and nitrate (bars = 20 μm). (B and D) SEM photomicrographs of strains CVO (B) and FWKO B (D) grown on sulfide and nitrate (bar = 2 μm).

designed for growth of lactate-oxidizing SRB were unsuccessful (results not shown).

Fermentation. Fermentative growth was not observed for strain CVO or FWKO B when tested on a variety of carbon substrates (Table 1), and neither CVO nor FWKO B grew in organic-rich, complex growth media designed for anaerobic growth of heterotrophic microorganisms.

Aerobic growth. Neither strain was capable of growth on sulfur, thiosulfate, or various carbon sources under fully aerobic (21% O_2) conditions (Table 1). However, autotrophic growth was observed for both strains under microaerophilic conditions (1% O_2), with CVO using either sulfide or elemental sulfur and FWKO B using hydrogen as an electron donor (Table 1).

Temperature, salts, and pH ranges. Growth of CVO occurred over a temperature range of 5°C (i.e., the lowest tested) to 35°C, a salinity range up to 7% NaCl, and a pH range from 5.5 to 8.5 (i.e., the highest tested). FWKO B grew over a temperature range of 10°C to 40°C, a salinity range of up to 3% NaCl, and a pH range from 6.0 to 8.5 (Table 3).

Sulfur and nitrogen transformation. Complete oxidation of 0.5 to 2.0 mM sulfide and 0.5 to 3.0 mM sulfide by CVO and FWKO B, respectively, occurred within 48 h under anaerobic conditions (Table 4). At the lower sulfide concentrations tested, CVO transformed 80 to 100% of the sulfide oxidized to SSC (Table 4), primarily in the form of sulfate, whereas at the higher sulfide concentrations tested, less than 15% of the sulfide oxidized was converted to SSC, suggesting that the majority of sulfide had been oxidized to elemental sulfur. However, only 29% of the sulfide-S, on average, was accounted for as elemental sulfur, while 61% of the total sulfur (Table 4, ΔS) remained unaccounted for. In contrast, oxidation of sulfide by FWKO B resulted in no increase in SSC regardless of the initial sulfide concentration. Instead, elemental sulfur accounted for 40 to 60% of the oxidized sulfide, on average, leaving the balance of the insoluble sulfur unaccounted for

TABLE 1. Major characteristics of strains CVO and FWKO B isolated from oil field brine

Parameter	Value[a]	
	CVO	FWKO B
Morphology	Slightly curved rods	Slightly curved rods
Width (μm)	0.4	0.4
Length (μm)	0.6–0.8	1.0–2.0
Spores	−	−
Motility	−[b]	+
Gram stain	−	−
Blood agar	−	−
Oxidase test	+	−
H_2O_2 decomposition	−	−
Aerobic growth		
Acetate, succinate, lactate, fumarate, malate, formate, pyruvate, glucose, glycerol	−	−
$S_2O_3^{2-}$	−	−
S^0	−	ND[d]
Microaerophilic growth (1% O_2)		
HS^-	+	+
S^0	+	ND
H_2	ND	+
Anaerobic growth on NO_3^- by oxidation of:		
HS^-	+	+
$S_2O_3^{2-}$	−	−
S^0	+	−
H_2	−	+
Formate	−	+
Acetate, pyruvate, succinate, fumarate, lactate, propionate, glucose	−	−
Anaerobic growth on NO_2 by oxidation of:		
HS^-	+	−
S^0	+	−
H_2	ND	−
Anaerobic growth on N_2O (2%) by oxidation of HS^-	+[c]	−
Anaerobic growth on H_2 by reduction of S^0	−	+
Anaerobic growth on acetate by reduction of S^0	ND	−
Growth by fermentation of pyruvate, succinate, lactate, fumarate, malate, aspartate	−	−

[a] +, positive; −, negative.
[b] Nonmotile or only weakly motile.
[c] Only a fivefold increase in cell numbers was observed after 5 days of incubation.
[d] ND, not determined.

(Table 4, ΔS). These sulfur values are likely underestimates of the true values, since extraction of elemental sulfur from biological cultures via solvent extraction can be problematic due to the propensity of sulfur to form aggregates with the biomass and adhere to surfaces. However, the fact that SSC did not increase further suggests that elemental sulfur was the sole end product.

At the lower sulfide concentrations tested, strain CVO converted over 60% of the nitrate reduced to nitrite, while less than 50% of the nitrate consumed was reduced to nitrite at the higher sulfide concentrations tested (2.0 and 3.0 mM). The nitrate-N unaccounted for is presumed to be in the form of nitrous oxide or dinitrogen gas. When strain CVO was added to CSB medium containing [^{15}N]nitrate (5 mM) and 3.0 mM sulfide, the production of $^{15}N_2O$ was detected but not that of $^{15}N_2$ (results not shown). Furthermore, there was no increase in ammonium. Growth of CVO did not occur, however, in CSB medium (0.5 mM sulfide) without nitrate in the presence of a headspace containing nitrous oxide (2%), nitrogen, and CO_2, suggesting that CVO is capable of denitrification. In contrast, nitrate reduction by FWKO B at all sulfide concentrations tested resulted in complete conversion to nitrite.

16S RNA oligonucleotide probe. A 16-base oligonucleotide probe (CLI) targeting a unique region of 16S rRNA from strain CVO did not display a strong hybridization signal with nucleic acids (primarily 16S RNA) from other sulfide-oxidizing bacteria (Fig. 3). However, a weaker hybridization signal, not visible in Fig. 3, was observed with *Thiomicrospira denitrificans*, while lengthening the probe to 21 bases apparently decreased the specificity so that both *Thiomicrospira denitrificans* and CVO displayed signals of equal intensity (results not shown). No hybridization was observed between the CLI probe and nucleic acids from strain FWKO B (Fig. 3). However, the CLI probe did strongly hybridize with nucleic acids from strain CVO, as expected, as well as nucleic acids from an enrichment of NR-SOB from produced brine collected at producing well 59-20. No hybridization was evident with nucleic acids from isolate Light C, which is an unidentified photosynthetic NR-SOB (possibly *Chromatium* sp.) isolated from CV-produced brine. As a positive control, it was shown that nucleic acids from all cell types hybridized with the general eubacterial probe EUB 338 (3).

Phylogenetic analysis. The top 100 hits of Blast searches with 16S rRNA gene sequences (positions 49 to 1313; *E. coli* numbering) of CVO, FWKO B, and *Campylobacter* sp. strain DSM 806 were all within the epsilon subdivision of the division *Proteobacteria*. Although these included matches with uncultured hydrothermal vent eubacteria and unidentified epsilon proteobacteria, only sequences of cultured and physiologically characterized representatives from this division were retrieved from GenBank. This included the best-matching sequences and sequences of representatives of genera within the epsilon

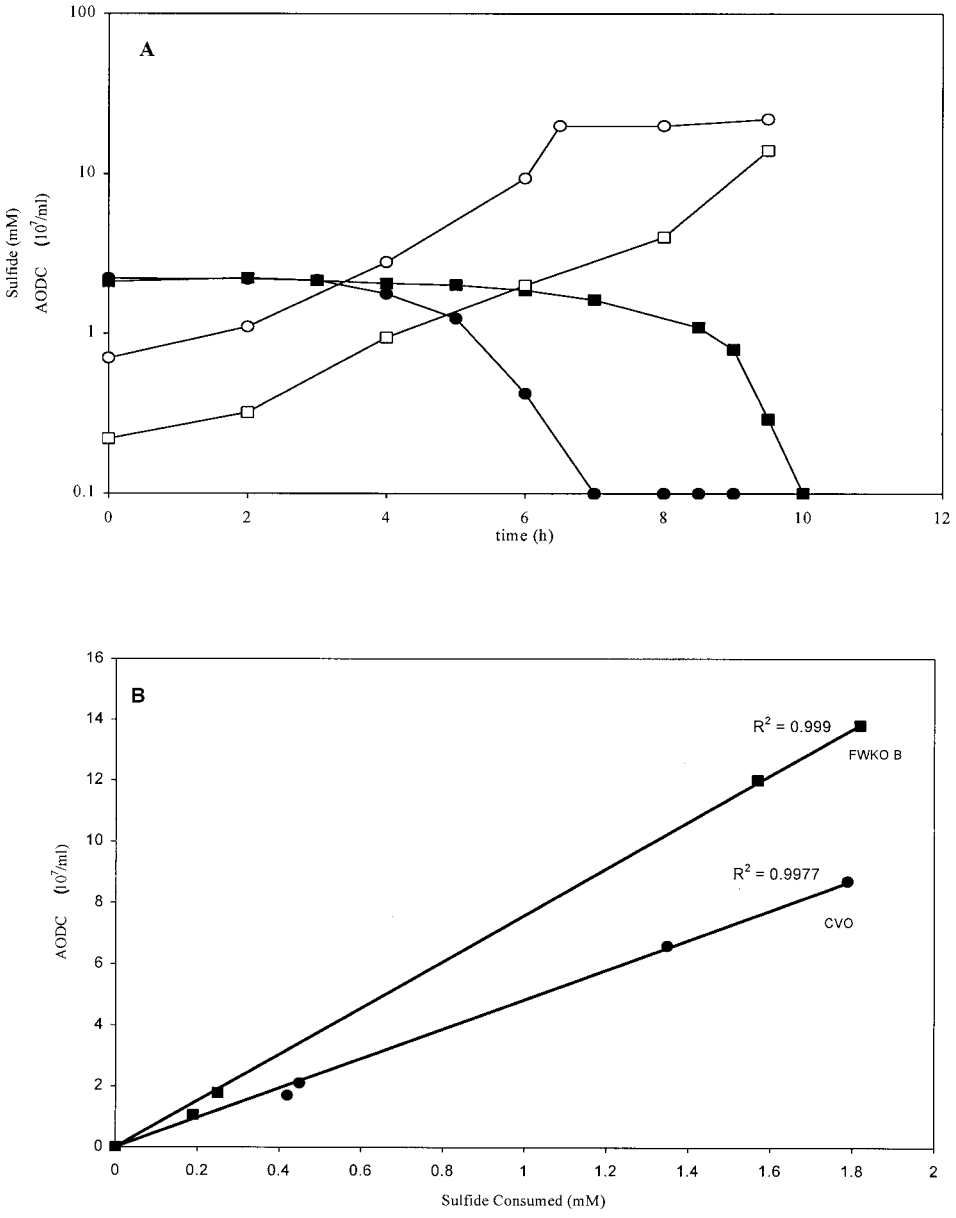


FIG. 2. (A) Growth curves and sulfide consumption rates, respectively, for CVO (○ and ●) and FWKO B (■ and □) growing at 30°C in DTA brine medium containing 2 mM sulfide and 10 mM KNO_3. Cell numbers are expressed as AODC. (B) Cell density versus sulfide consumption for CVO and FWKO B plotted from data in panel A. Linear trend lines and R^2 values were determined with Microsoft Excel 97 by setting the *y* intercept to 0.

subdivision. Pairwise sequence similarities calculated with the GCG program GAP ranged from 88 to 99%. The PileUp dendrogram derived from all pairwise similarity coefficients is shown in Fig. 4A. The 16S rRNA sequence of CVO was most similar to that of *Thiomicrospira denitrificans* (96.1% similarity), while the sequence of FWKO B was most closely related to that of *Arcobacter* spp. (92% similarity) and *Sulfurospirillum* spp. (90% similarity). The sequence obtained for *Campylobacter* sp. strain DSM 806 was nearly identical to that of *Campylobacter* sp. strain CCUG 1392 CLO. Both of these environmental *Campylobacter* spp. are most closely related to members of the newly defined genus *Sulfurospirillum*. The phylogenetic tree obtained with PAUP (Fig. 4B) closely matches

TABLE 2. Growth of strains CVO and FWKO B on organic or inorganic carbon sources in the presence of various electron donors and acceptors

Strain/donor/acceptor	Growth on carbon source[a]		
	CO_2-acetate	CO_2	Acetate
CVO/HS^-/NO_3^-	+	+	±
CVO/S^0/NO_3^-	+	ND	ND
FWKO B/HS^-/NO_3^-	+	+	−
FWKO B/H_2/S^0	+	+	ND

[a] ND, not determined; +, growth; −, no growth; ±, as much as 0.4 mM bicarbonate was transferred with the inoculum (2%) such that at least some of the growth yield (10^7 cells/ml) might have been due to assimilated HCO_3^-.

TABLE 3. Effects of temperature, salinity, and pH on growth of strains CVO and FWKO B

Growth conditions[a]	Growth[b]	
	CVO	FWKO B
5°C	+	−
10°C	+	+
35°C	+	+
40°C	−	+
45°C	−	−
0% NaCl	+	+
1.0% NaCl	+	+
3.0% NaCl	+	+
5.0% NaCl	+	−
7.0% NaCl	+	−
10.0% NaCl	−	ND
pH 5.5	+	−
pH 6.0	+	±[c]
pH 7.0	+	+
pH 8.5	+	+

[a] Growth in CSB medium with 2 to 3 mM sulfide for temperature tests and 1 mM sulfide for salinity and pH tests. All tests were run in triplicate except where noted in the text.
[b] ND, not determined; +, growth; −, no growth.
[c] Complete oxidation of sulfide was noted in only one of two replicates.

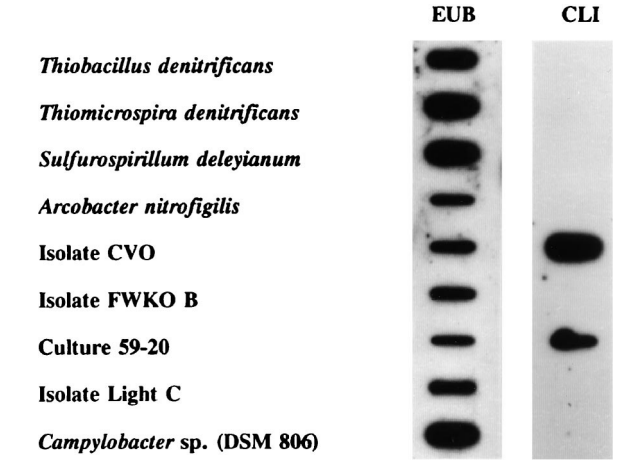


FIG. 3. Hybridization of either labeled eubacterial (EUB 338) or labeled CVO-specific (CLI) oligonucleotide probe to nucleic acids from whole cells of bacteria known to be closely related to CVO (Fig. 4; isolate Light C is described in the text) or to nucleic acids from cells from an enrichment of sulfide-laden produced brine collected at CV Well 59-20 amended with nitrate and phosphate (culture 59-20).

that shown in Fig. 4A. Most branches are supported by high bootstrap values.

DISCUSSION

Phylogeny. Earlier analysis of a shorter 16S rRNA sequence for CVO indicated its classification as having the closest homology with *Campylobacter* sp. strain CCUG 1392 CLO (70). Since then, the genus *Sulfurospirillum* has been defined (17, 52) and a detailed analysis of the phylogeny of the genus *Thiomicrospira* has appeared (42). The epsilon subdivision of the division *Proteobacteria* currently comprises the *Campylobacter* group (genera *Arcobacter* and *Campylobacter*), the *Helicobacter* group (genera *Flexispira*, Gastrospirillum, *Helicobacter*, *Thiovulum*, and *Wolinella*), the genus *Sulfurospirillum*, and unclassified epsilon *Proteobacteria* which include *Thiomicrospira denitrificans* (http://www.ncbi.nlm.nih.gov/Taxonomy/tax/html). The genus *Sulfurospirillum* includes species previously indicated to be members of the genus *Geospirillum* (*Sulfurospirillum barnesii*, *Sulfurospirillum arsenophilum*, and *Sulfurospirillum* sp. strain SM-5 [59]) as well as *Dehalospirillum multivorans* (51), *Campylobacter* sp. strain CCUG 1392 CLO, and *Campylobacter* sp. strain DSM 806. All of these have high (97 to 99%) 16S rRNA sequence similarity to *S. deleyianum* (17), the type strain of the genus, as shown in Fig. 4A and have comparable morphological and physiological properties (52). Reclassification of *Campylobacter* spp. strains CCUG 1392 CLO and DSM 806 as *Sulfurospirillum* spp. makes the *Campylobacter* clade physiologically more homogeneous, i.e., strictly free-living organisms are now excluded and the remaining *Campylobacter* spp. all serve as human or animal pathogens. Strain CVO is a close relative of neither the *Sulfurospirillum* spp. nor the newly defined clade of *Campylobacter* spp. (Fig. 4); instead, it most closely resembles *Thiomicrospira denitrificans*. The genus *Thiomicrospira* has members in both the gamma (*Thiomicrospira crunogena*, *Thiomicrospira pelophila*, and *Thiomicrospira* sp. strain MA2-6) and epsilon (*Thiomicrospira denitrificans* only) subdivisions of proteobacteria (42zrefx. Strain CVO is thus the second well-characterized *Thiomicrospira* sp.

TABLE 4. Transformation of nitrate-N (10 mM) and sulfide-S (0.5 to 3.0 mM) during sulfide oxidation by strains CVO and FWKO B

Initial HS^- (mM)	ΔHS^- (mM)	ΔSSC[a] (mM)	ΔSO_4^{2-} (mM)	S^0 (mM)	ΔS[d] (mM)	ΔNO_3^- (mM)	ΔNO_2^- (mM)	ΔN[e] (mM)
Strain CVO								
0.5	−0.5 ± 0.0[b]	0.5 ± 0.00	0.3 ± 0.12	0.04 ± 0.04	−0.16	−2.2 ± 0.50	1.5 ± 0.10	−0.7
1.0	−1.0 ± 0.0	0.8 ± 0.01	0.8 ± 0.10	0.02 ± 0.01	−0.18	−3.3 ± 0.38	2.2 ± 0.29	−1.1
2.0	−2.0 ± 0.0	0.3 ± 0.24	0.2 ± 0.18	0.58 ± 0.18	−1.22	−1.7 ± 0.40	0.66 ± 0.20	−1.0
3.0	−2.9 ± 0.16	0.0[c]	0.2[c]	ND	ND	−2.8[c]	1.1[c]	−1.7
Strain FWKO B								
0.5	−0.5 ± 0.0[b]	−0.4 ± 0.12	−0.2 ± 0.17	0.26 ± 0.04	−0.24	−0.9 ± 0.32	0.88 ± 0.03	0
1.0	−1.0 ± 0.0	−0.3 ± 0.0	0.0 ± 0.0	0.57 ± 0.08	−0.43	−1.2 ± 0.10	1.4 ± 0.15	0
2.0	−2.0 ± 0.0	−0.4 ± 0.12	−0.2 ± 0.12	1.15 ± 0.09	−0.85	−2.3 ± 0.31	2.3 ± 0.06	0
3.0	−3.0 ± 0.0	−0.3 ± 0.25	0.0 ± 0.06	1.37 ± 0.04	−1.63	−3.2 ± 0.06	3.0 ± 0.08	0

[a] Soluble sulfur as measrued by X-ray fluroescence spectroscopy.
[b] Samples after 48 h of incubation at 30°C; average ± standard deviation; $n = 3$.
[c] $n = 1$.
[d] Total sulfur unaccounted for as either SO_4^{2-} or S^0.
[e] Total nitrogen unaccounted for in the form of either N_2O or N_2; calculated as $\Delta NO_3^- + \Delta NO_2^-$.

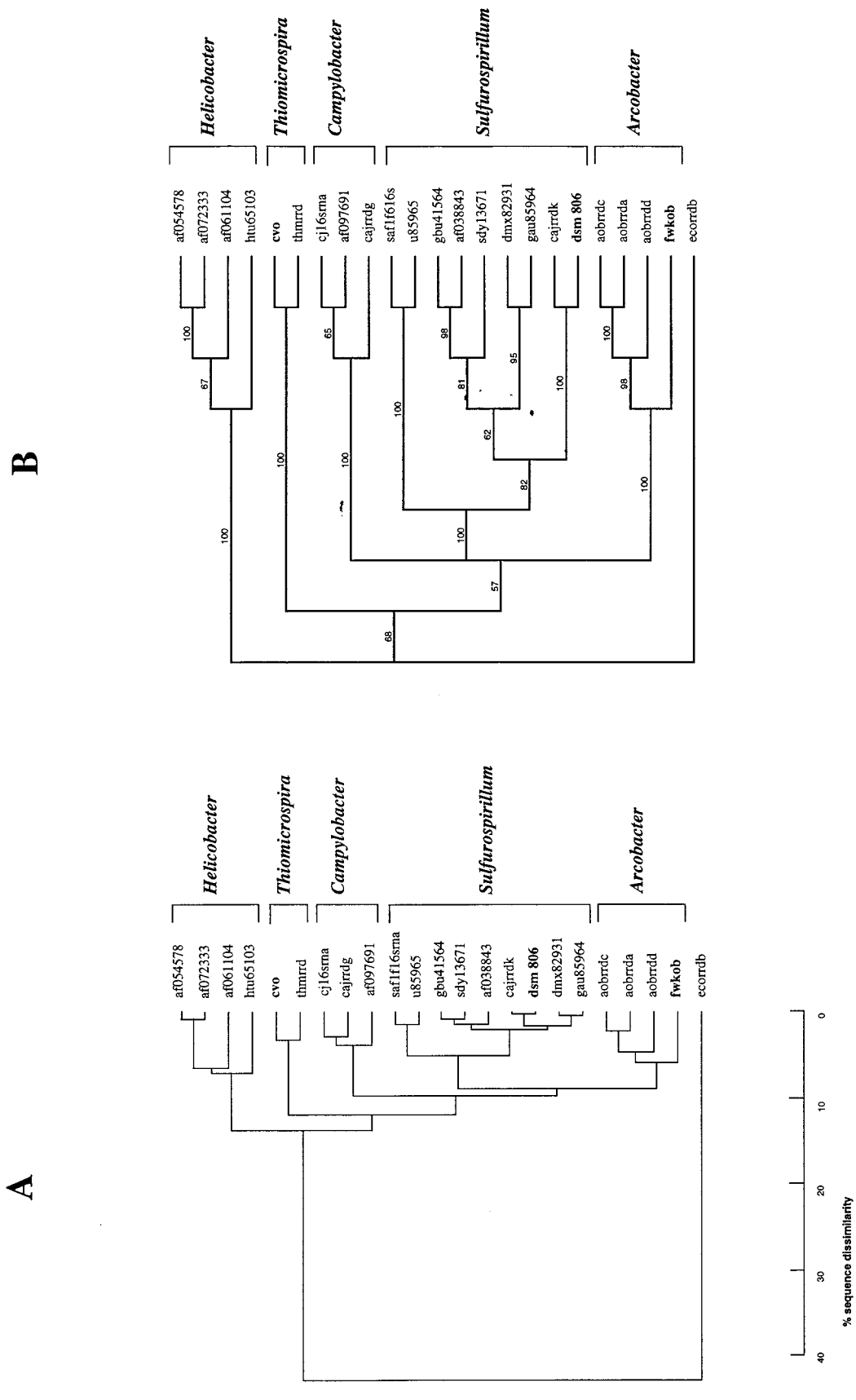


FIG. 4. Phylogenetic trees of 16S rRNA sequences of strains CVO and FWKO B. (A) Tree based on pairwise homologies obtained with the GCG program PileUp. The horizontal distances reflect the pairwise sequence similarities as indicated on the scale. (B) Tree obtained by PAUP analysis with the sequence for *E. coli* as the outgroup and using 100 bootstrap replicates. The observed frequencies of the groups are indicated at the nodes. The horizontal distances are arbitrary. In addition to the sequences for CVO and FWKO B, the following sequences are represented in the tree: af054578, *Helicobacter* sp. strain UNSW1.7sp; af072333, *Helicobacter* sp. strain hamster B; af061104, *Helicobacter typhlonicus*; htu65103, *Helicobacter trogontum*; thmrrd, *Thiomicrospira denitrificans*; cj16srna, *Campylobacter jejuni*; cajrrdg, *Campylobacter upsaliensis*; af097691, *Campylobacter hyointestinalis*; saf1f16srna, *Sulfurospirillum arcachonense*; u85965, *Sulfurospirillum* sp. strain SM-5; gbu41564, *S. barnesii*; sdy13671, *S. deleyianum*; af038843, *S. barnesii*; cajrrdk, *Campylobacter* sp. strain CCUG 1392 CLO; dsm806, *Campylobacter* sp. strain DSM806 (determined in this study); dmx82931, *D. multivorans*; gau85964, *S. arsenophilum*; aobrrdc, *A. butzleri*; aobrrda, *A. cryoaerophilus*; aobrrdd, *A. nitrofigilis*; ecorrdb, *E. coli*. The strains sequenced in this study are in boldface.

in the epsilon subdivision. We propose to rename the organism *Thiomicrospira* sp. strain CVO from *Campylobacter* sp. strain CVO, the name proposed earlier (70).

From the dendrograms in Fig. 4, it appears that FWKO B is most closely affiliated with members of the genus *Arcobacter*, which includes both free-living (*A. nitrofigilis*) and pathogenic (e.g., *Arcobacter butzleri* and *Arcobacter cryaerophilus*) species. FWKO B forms a deep branch in the *Arcobacter* clade and has some distinct physiological properties. However, the branching is not deeper than that in the genus *Helicobacter* (Fig. 4A), and the designation *Arcobacter* sp. strain FWKO B is, therefore, appropriate.

Phenotypic characteristics of CVO. *Thiomicrospira denitrificans* and *Thiomicrospira* sp. strain CVO are obligate chemolithoautotrophs. Like *Thiomicrospira* sp. strain CVO, *Thiomicrospira denitrificans* oxidizes sulfide with nitrate as the electron acceptor. *Thiomicrospira denitrificans*, and probably also *Thiomicrospira* sp. strain CVO (see below), denitrifies nitrate to nitrogen. *Thiomicrospira denitrificans* oxidizes sulfide to sulfate (65), whereas *Thiomicrospira* sp. strain CVO forms sulfur and sulfate, depending on the sulfide-to-nitrate ratio. However, *Thiomicrospira denitrificans* also used thiosulfate as an electron donor, an activity that is not shared by *Thiomicrospira* sp. strain CVO (Table 1). *Thiomicrospira denitrificans* also has a distinct morphology, forming long spirals of 4 to 5 μm (36), whereas strain CVO forms only very small curved rods less than 1 μm in length (Fig. 1). The CVO-specific oligonucleotide probe CL1 reacted only weakly with nucleic acids from *Thiomicrospira denitrificans* due to the presence of two mismatches.

In contrast to this strict chemolithoautotrophic growth of strain CVO (Table 1), sulfurospirillae can grow as chemoorganoheterotrophs using organic acids or amino acids as carbon and energy sources. Growth by reduction of fumarate to succinate is a characteristic of *S. deleyianum* and strain DSM 806, supporting their close affiliation (Fig. 4), but not of strain CVO. *S. deleyianum* but not *Thiomicrospira* sp. strain CVO can use elemental sulfur as an electron acceptor. The similarities are that all are curved or vibroid, grow only microaerophilically or anaerobically, are oxidase positive, and do not ferment glucose or reduce sulfate.

S. deleyianum dissimilates nitrate to ammonium (52), whereas CVO reduces nitrate to nitrous oxide and presumably dinitrogen: the growth of CVO in the presence of sulfide (0.5 mM) and nitrous oxide (2%) suggests that nitrogen is produced. The accumulation of nitrous oxide in the presence of 3.0 mM sulfide and nitrate indicates inhibition of nitrous oxide reductase by sulfide. As little as 0.3 mM H_2S resulted in almost total inhibition of nitrous oxide reduction by denitrifying bacteria (54). Gram-negative aerobic and microaerobic denitrifiers reported to produce nitrous oxide as an end product in the absence of sulfide have also been described (74). A strictly anaerobic, thermophilic, chemolithoautotrophic archaeum, *Ferroglobus placidus*, dissimilates nitrate to nitrous oxide while oxidizing either sulfide, H_2, or ferrous iron (71). The colorless sulfur-oxidizing bacteria of the genera *Thiobacillus*, *Thermothrix*, *Thiosphaera*, and *Thiomicrospira* are also capable of denitrification, but unlike CVO, they also oxidize thiosulfate as an alternate energy source.

Phenotypic characteristics of *Arcobacter* sp. strain FWKO B. *Arcobacter* spp., like FWKO B, are gram-negative, motile, curved rods. They do not ferment glucose, and they grow at temperatures between 15 and 40°C, reduce nitrate, and grow under microaerophilic conditions (38, 39, 64, 68). However, unlike other *Arcobacter* spp., FWKO B is catalase and oxidase negative. In addition, *Arcobacter* spp. are chemoorganotrophs unable to grow lithotrophically, unlike FWKO B, which is a chemolithotroph capable of oxidizing sulfide and hydrogen but incapable of using any of the organic compounds tested, except formate, as an energy source (Table 1). Also, FWKO B grows autotrophically and could not use acetate as a carbon source in the absence of CO_2, while *Arcobacter* spp. use amino acids and other organics as carbon sources (68). Furthermore, FWKO B is a hydrogenotrophic sulfur reducer, whereas hydrogen is reported to only stimulate growth of *Arcobacter* spp. (67). Widdel and Pfennig (72) indicated that growth by hydrogenotrophic, sulfur-reducing eubacteria typically requires 1 to 2 mM acetate in addition to CO_2. Although several thermophilic chemolithoautotrophic sulfur reducers from the domain *Archaea* (25, 53) and several from the domain *Bacteria* (21) have been reported, FWKO B represents the only mesophilic chemolithoautotrophic sulfur reducer in the domain *Bacteria*.

Although both *A. nitrofigilis* and FWKO B reduce nitrate only to nitrite, FWKO B grows (Fig. 2) but *A. nitrofigilis* does not, due to the toxicity of the nitrite formed (38). Certain members of the neutrophilic, chemolithotrophic sulfur bacteria also produce nitrite and typically require the presence of a nitrite-reducing bacterium for growth in the presence of nitrate (49).

Growth and sulfur production by CVO and FWKO B. Growth was proportional to sulfide oxidation for both CVO and FWKO B, indicating that both are capable of electron transport from sulfide to nitrate (Fig. 2B). The doubling times of 1.3 and 1.5 h for CVO and FWKO B, respectively, are significantly lower than the minimum doubling time of 2.85 h reported for the aerobic chemolithoautotrophic sulfide oxidizers *Thiobacillus neapolitanus* and *Thiobacillus* sp. strain O (29).

Both CVO and FWKO B oxidized sulfide to elemental sulfur, but only CVO was capable of complete oxidation to sulfate, which was dependent on the nitrate-to-sulfide ratio (Table 4). A shift from sulfate to sulfur production was also observed for two aerobic sulfide-oxidizing *Thiobacillus* spp. at increasing sulfide concentrations (29, 67, 69). Sulfur production by aerobic, sulfide-oxidizing thiobacilli was determined to be maximal at an oxygen/sulfide uptake ratio near the expected theoretical value of 0.56 (69). Sulfur production by CVO occurred at a nitrate/sulfide ratio of 0.52 (Table 4), which is also close to its expected theoretical ratio when nitrous oxide is the end product: $HS^- + 0.5\ NO_3^- + 1.5\ H^+ \rightarrow S^0 + 0.25\ N_2O + 1.25\ H_2O$. Therefore, CVO responds similarly to the aerobic thiobacilli by producing elemental sulfur when nitrate is limiting.

S. deleyianum oxidizes sulfide to elemental sulfur while reducing nitrate to ammonium (15). Its ability to shift electrons from production of sulfur to production of sulfate cannot be evaluated, since the tests were conducted in the presence of high sulfide (4 to 5 mM) and limiting nitrate (1.2 and 5 mM) concentrations. Elemental sulfur was also the end product of fumarate-dependent sulfide oxidation by *Wolinella succinogenes* and *S. deleyianum* (37, 73).

Ecological significance. Colorless sulfur bacteria are widespread in nature and have been isolated from sulfur deposits, hot sulfur springs, soils, freshwater, and seawater (35). Bharathi et al. (6) isolated over 100 strains of anaerobic, colorless NR-SOB from seawater and a sulfide-laden creek. However, few reports have been made of the isolation of NR-SOB from petroleum reservoir brines, even though their activity has been observed (28, 41, 55, 61, 70). Dannenberg et al. (13) reported that the SRB *Desulfobulbus propionicus* also displays NR-SOB activity, but its presence in oil field brines has not been reported.

Sulfide-oxidizing microaerophiles such as CVO might utilize limiting amounts of oxygen or nitrate diffusing from surface

water or shallow groundwater to oxidize sulfide produced by indigenous SRB in petroleum reservoirs (63, 70). Cycling of sulfur in oil reservoirs, such as CV, likely occurs between sulfide and elemental sulfur in view of the high concentrations of sulfide (>3 mM), which favor the formation of elemental sulfur (Table 4) (30).

Since CVO is the dominant NR-SOB in CV brine amended with nitrate (61, 63), the role of FWKO B may be that of a sulfur reducer and not an NR-SOB. Pfennig and Biebl (46) reported the cycling of sulfide and elemental sulfur by a phototrophic green sulfur bacterium and the acetate-oxidizing sulfur reducer *Desulfuromonas acetoxidans*. Likewise, efficient cycling of sulfide and elemental sulfur by *D. acetoxidans* and CVO has been demonstrated (63).

The ability of FWKO B and CVO to grow lithoautotrophically with sulfide and nitrate suggests the potential for a subterranean sulfur cycle driven solely by the inorganic nutrients hydrogen, nitrate, and CO_2. We recently demonstrated that cycling between sulfide and elemental sulfur occurs in a CV brine enrichment by alternating the addition of nitrate and hydrogen gas to the enrichment (results not shown). Hydrogen gas in this shallow reservoir could be generated from fermentation or by geochemical mechanisms (34, 57, 58). Bicarbonate ions are plentiful in this sandstone reservoir brine (approximately 23 mM), while nitrates, used as fertilizer in this agricultural area, could enter the reservoir by diffusion from nitrate-contaminated surface waters or via injection of nitrate-contaminated groundwater used for improving oil recovery. Alternatively, the presence of these lithoautotrophic NR-SOB in oil field waters provides opportunities for the application of biologically mediated removal of sulfides in oil field waters through the simple addition of inexpensive nutrients, such as nitrate and phosphate (28, 33, 41).

ACKNOWLEDGMENTS

This work was supported by Phillips Petroleum Company and was performed as an in-kind contribution to the U.S. DOE-funded project Bioreactor Design and Demonstration for Microbial Oxidation of Sulfides under contract DOE AC-10-15 and under contract FWP 4340-42, which is being conducted under a Cooperative Research and Development Agreement (99-CR-01) with The Idaho National Engineering and Environmental Laboratory, Idaho Falls, Idaho. Research in G.V.'s laboratory was supported by a Strategic Grant from the Natural Science and Engineering Research Council of Canada (NSERC).

We thank Miriam Wright, Glenna Thompson, Robert H. Webb, and Michael Moradi-Araghi for their technical assistance. We also thank Michael Madigan for his helpful comments.

REFERENCES

1. **Allen, L. A.** 1949. The effect of nitro-compounds and some other substances on production of hydrogen sulfide by sulphate-reducing bacteria in sewage. Proc. Soc. Appl. Bacteriol. **2:**26–38.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Amann, R. I., J. Stromley, R. Devereux, R. Key, and D. A. Stahl.** 1992. Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. Appl. Environ. Microbiol. **58:**614–623.
4. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique group. Microbiol. Rev. **43:**260–296.
5. **Bentzen, G., A. T. Smith, D. Bennett, N. J. Webster, F. Reinholt, E. Sletholt, and J. Hobson.** 1995. Controlled dosing of nitrate for prevention of H_2S in a sewer network and the effects on the subsequent treatment processes. Water Sci. Technol. **31:**293–302.
6. **Bharathi, P. A. L., S. Nair, and D. Chandramohan.** 1997. Anaerobic sulfide-oxidation in marine colorless sulfur-oxidizing bacteria. Mar. Biotechnol. **5:**172–177.
7. **Braun-Howland, E. B., P. A. Vescio, and S. A. Nierzwicki-Bauer.** 1993. Use of a simplified cell blot technique and 16S RNA directed probes for identification of common environmental isolates. Appl. Environ. Microbiol. **59:** 3219–3224.
8. **Carpenter, W. T.** 1932. Sodium nitrate used to control nuisance. Water Works Sewage **79:**75–176.
9. **Chan, C. W., and I. Suzuki.** 1993. Quantitative extraction and determination of elemental sulfur and stoichiometric oxidation of sulfide to elemental sulfur by *Thiobacillus thiooxidans*. Can. J. Microbiol. **39:**1166–1168.
10. **Collins, C. H., and P. M. Lyne.** 1976. Microbiological methods. Butterworth & Co. Ltd., London, United Kingdom.
11. **Costilow, R. N.** 1981. Biophysical factors in growth, p. 66–78. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
12. **Craig, F. F., Jr.** 1971. Reservoir engineering aspects of waterflooding. Monograph series. Society of Petroleum Engineers, Dallas, Tex.
13. **Dannenberg, S., M. Kroder, W. Dilling, and H. Cypionka.** 1992. Oxidation of H_2, organic compounds and inorganic sulfur compounds coupled to reduction of O_2 or nitrate by sulfate-reducing bacteria. Arch. Microbiol. **158:**93–99.
14. **Doetsch, R. N.** 1981. Determinative methods of light microscopy, p. 21–33. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
15. **Eisenmann, E., J. Beurle, K. Sulger, P. M. H. Kronek, and W. Schumacher.** 1995. Lithotrophic growth of *Sulfurospirillum deleyianum* with sulfide as electron donor coupled to respiratory reduction of nitrate to ammonia. Arch. Microbiol. **164:**180–185.
16. **Fales, A. L.** 1929. Treatment of industrial wastes from paper mills and tannery on Neponsit River. J. Ind. Eng. Chem. **21:**216.
17. **Finster, K., W. Liesack, and B. J. Tindall.** 1997. *Sulfurospirillum arcachonense* sp. nov., a new microaerophilic sulfur-reducing bacterium. Int. J. Syst. Bacteriol. **47:**1212–1217.
18. **Fogo, J. K., and M. Popowski.** 1949. Spectrophotometric determination of hydrogen sulfide. Anal. Biochem. **21:**732–734.
19. **Gevertz, D., G. E. Jenneman, S. Zimmerman, and J. Stevens.** 1995. Microbial oxidation of soluble sulfides in produced water from the Bakken sands, p. 295–310. *In* R. Byrant and K. L. Sublette (ed.), Proceedings of the 5th International Conference on Microbial Enhanced Oil Recovery and Related Biotechnology for Solving Environmental Problems. National Technical Information Services, Springfield, Va.
20. **Gherna, R., P. Pienta, and R. Cote (ed.).** 1992. ATCC catalogue of bacteria & bacteriophages. American Type Culture Collection, Rockville, Md.
21. **Hedderich, R., O. Klimmek, A. Kroger, R. Dirmeier, M. Keller, and K. O. Stetter.** 1999. Anaerobic respiration with elemental sulfur and with disulfides. FEMS Microbiol. Rev. **22:**353–381.
22. **Heukelekian, H.** 1943. Effect of the addition of sodium nitrate to sewage on hydrogen sulfide production and B.O.D. reduction. Sewage Works J. **15:**255–261.
23. **Hicks, R. E., R. I. Amann, and D. A. Stahl.** 1992. Dual staining of natural bacterioplankton with 4′, 6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. Appl. Environ. Microbiol. **58:**2158–2163.
24. **Hitzman, D. O., G. T. Sperl, and K. A. Sandbeck.** April 1995. U.S. patent 5,405,593.
25. **Huber, R., and K. O. Stetter.** 1991. The order Thermoproteales, p. 677–683. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed., vol. I. Springer-Verlag, New York, N.Y.
26. **Hutchinson, M., K. I. Johnstone, and D. White.** 1967. The taxonomy of anaerobic *Thiobacilli*. J. Gen. Microbiol. **47:**17–23.
27. **Jack, T. R., E. Lee, and J. Mueller.** 1985. Anaerobic gas production: controlling factors, p. 167–180. *In* J. E. Zajic and E. C. Donaldson (ed.), Microbes and oil recovery. Proceedings of the International Conference on Microbial Enhancement of Oil Recovery. Petroleum Bioresources, El Paso, Tex.
28. **Jack, T. R., and D. W. S. Westlake.** 1995. Control in industrial settings, p. 265–292. *In* L. L. Barton (ed.), Sulfate-reducing bacteria. Plenum Press, New York, N.Y.
29. **Janssen, A. J. H., R. Sleyster, C. van der Kaa, A. Jochemsen, J. Bontsema, and G. Lettinga.** 1995. Biological sulphide oxidation in a fed-batch reactor. Biotechnol. Bioeng. **47:**327–333.
30. **Jenneman, G. E., and D. Gevertz.** Identification, characterization, and application of sulfide-oxidizing bacteria in oil fields. *In* Proceedings of the 8th International Meeting on Microbial Ecology, Halifax, Nova Scotia, in press.
31. **Jenneman, G. E., D. Gevertz, and M. Wright.** 1996. Sulfide bioscavenging of soured produced water by natural microbial populations, p. 693–701. *In* Proceedings of the 3rd International Petroleum Environmental Conference, Albuquerque, N.M. [CD-ROM.]
32. **Jenneman, G. E., M. J. McInerney, and R. M. Knapp.** 1986. Effect of nitrate on biogenic sulfide production. Appl. Environ. Microbiol. **51:**1205–1211.
33. **Jenneman, G. E., P. D. Moffitt, G. A. Bala, and R. H. Webb.** 1999. Sulfide removal in reservoir brine by indigenous bacteria. Soc. Petroleum Eng. Prod. Facilities **14:**219–225.
34. **Krumholtz, L. R.** 1998. Microbial ecosystems in the Earth's subsurface. ASM News **64:**197–202.
35. **Kuenen, J. G.** 1975. Colourless sulfur bacteria and their role in the sulfur cycle. Plant Soil **43:**49–76.

36. **Kuenen, J. G., L. A. Robertson, and O. H. Tuovinen.** 1991. The genera *Thiobacillus*, *Thiomicrospira*, and *Thiosphaera*, p. 2638–2657. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed., vol. III. Springer-Verlag, New York, N.Y.
37. **Macy, J. M., I. Schroder, R. K. Thauer, and A. Kroger.** 1986. Growth of *Wolinella succionogenes* on H_2S plus fumarate and on formate plus sulfur as energy sources. Arch. Microbiol. **144:**147–150.
38. **McClung, C. R., and D. G. Patriquin.** 1980. Isolation of a nitrogen-fixing *Campylobacter* species from the roots of *Spartina alterniflora* Loisel. Can. J. Microbiol. **26:**881–886.
39. **McClung, C. R., D. G. Patriquin, and R. E. Davis.** 1983. *Campylobacter nitrofigilis* sp. nov., a nitrogen-fixing bacterium associated with roots of *Spartina alterniflora* Loisel. Int. J. Syst. Bacteriol. **33:**605–612.
40. **McInerney, M. J., and K. L. Sublette.** 1997. Petroleum microbiology: biofouling, souring and improved oil recovery, p. 600–607. *In* C. J. Hurst, G. R. Knudsen, M. J. McInerney, L. D. Stetzenbach, and M. V. Walter (ed.), Manual of environmental microbiology. American Society for Microbiology, Washington, D.C.
41. **McInerney, M. J., K. L. Sublette, V. K. Bhuparthiaraju, J. D. Coates, and R. M. Knapp.** 1993. Causes and control of microbially induced souring, p. 363–371. *In* E. T. Premuzic and A. Woodhead (ed.), Developments in petroleum science. 39. Microbial enhancement of oil recovery—recent advances. Proceedings of the 1992 International Conference on Microbial Enhanced Oil Recovery. Elsevier, Amsterdam, The Netherlands.
42. **Muyzer, G., A. Teske, C. O. Wirsen, and H. W. Jannasch.** 1995. Phylogenetic relationships of Thiomicrospira species and their identification in deep sea hydrothermal vent samples by denaturing gradient electrophoresis of 16S rRNA fragments. Arch. Microbiol. **164:**165–172.
43. **Nelson, D.C.** 1991. The genus *Beggiatoa*, p. 3171–3180. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed., vol. IV. Springer-Verlag, New York, N.Y.
44. **Olsen, G. J., D. J. Lane, S. J. Giovannoni, N. R. Pace, and D. A. Stahl.** 1986. Microbial ecology and evolution: a ribosomal RNA approach. Annu. Rev. Microbiol. **40:**337–365.
45. **Okabe, S., W. L. Jones, W. Lee, and W. G. Characklis.** 1994. Anaerobic SRB biofilms in industrial water systems: a process analysis, p. 189–204. *In* G. G. Geesey, Z. Lewandowski, and H.-C. Flemming (ed.), Biofouling and biocorrosion in industrial water systems. Lewis Publishers, Boca Raton, Fla.
46. **Pfennig, N., and H. Biebl.** 1976. *Desulfuromonas acetoxidans* gen. nov. and sp. nov., a new anaerobic, sulfur-reducing, acetate-oxidizing bacterium. Arch. Microbiol. **110:**3–12.
47. **Poduska, R. A., and B. D. Anderson.** 1981. Successful storage lagoon odor control. J. Water Pollut. Control Fed. **53:**299–310.
48. **Reinsel, M. A., J. T. Sears, P. S. Stewart, and M. J. McInerney.** 1996. Control of microbial souring by nitrate, nitrite or glutaraldehyde injection in a sandstone column. J. Ind. Microbiol. **17:**128–136.
49. **Robertson, L. A., and J. G. Kuenen.** 1991. The colorless sulfur bacteria, p. 385–413. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed., vol. I. Springer-Verlag, New York, N.Y.
50. **Sandbeck, K. A., and D. O. Hitzman.** 1995. Biocompetitive exclusion technology: a field system to control reservoir souring and increase production, p. 311–320. *In* R. Byrant and K. L. Sublette (ed.), Proceedings of the 5th International Conference on Microbial Enhanced Oil Recovery and Related Biotechnology for Solving Environmental Problems. National Technical and Information Services, Springfield, Va.
51. **Scholz-Muramatsu, H., A. Neumann, M. Messmer, E. Moore, and G. Diekert.** 1995. Isolation and characterization of *Dehalospirillum multivorans* gen. nov., sp. nov., a tetrachloroethene-utilizing, strictly anaerobic bacterium. Arch. Microbiol. **163:**48–56.
52. **Schumacher, W., P. M. H. Kroneck, and N. Pfennig.** 1992. Comparative systematic study on "Spirillum" 5175, *Campylobacter* and *Wolinella* species. Arch. Microbiol. **158:**287–293.
53. **Segerer, A. H., and K. O. Stetter.** 1991. The order Sulfobales, p. 684–701. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed., vol. I. Springer-Verlag, New York, N.Y.
54. **Sørensen, J., J. M. Tiedje, and R. B. Firestone.** 1980. Inhibition by sulfide of nitric and nitrous oxide by denitrifying *Pseudomonas fluorescens*. Appl. Environ. Microbiol. **39:**105–110.
55. **Sperl, G. T., P. L. Sperl, and D. O. Hitzman.** 1993. A use of natural microflora, electron acceptors and energy sources for enhanced oil recovery, p. 17–25. *In* E. T. Premuzic and A. Woodhead (ed.), Developments in petroleum science. 39. Microbial enhancement of oil recovery—recent advances. Proceedings of the 1992 International Conference on Microbial Enhanced Oil Recovery. Elsevier, Amsterdam, The Netherlands.
56. **Staden, R.** 1987. Computer handling of DNA sequencing projects, p. 173–217. *In* M. J. Bishop and C. J. Rawlings (ed.), Nucleic acid and protein sequence analysis. A practical approach. IRL Press, Oxford, United Kingdom.
57. **Stevens, T.** 1997. Lithoautotrophy in the subsurface. FEMS Microbiol. Rev. **20:**327–337.
58. **Stevens, T. O., and J. P. McKinley.** 1995. Lithoautotrophic microbial ecosystems in deep basalt aquifers. Science **270:**450–454.
59. **Stolz, J. F., D. J. Ellis, J. Switzer Blum, D. Ahmann, D. R. Lovley, and R. S. Oremland.** 1999. *Sulfurospirillum barnesii* sp. nov. and *Sulfurospirillum arsenophilum* sp. nov., new members of the Sulfurospirillum clade of the epsilon Proteobacteria. Int. J. Syst. Bacteriol. **49:**1177–1180.
60. **Swofford, D. L.** 1993. Phylogenetic analysis using parsimony, version 3.1. Illinois Natural History Survey, Champaign, Ill.
61. **Telang, A. J., S. Ebert, J. M. Foght, D. W. S. Westlake, G. E. Jenneman, D. Gevertz, and G. Voordouw.** 1997. Effect of nitrate injection on the microbial community in an oil field as monitored by reverse genome probing. Appl. Environ. Microbiol. **63:**1785–1793.
62. **Telang, A. J., S. Ebert, J. M. Foght, D. W. S. Westlake, and G. Voordouw.** 1998. Effects of two diamine biocides on the microbial community from an oil field. Can. J. Microbiol. **44:**1060–1065.
63. **Telang, A. J., G. E. Jenneman, and G. Voordouw.** 1999. Sulfur cycling in mixed cultures of sulfide-oxidizing and sulfate- or sulfur-reducing oil field bacteria. Can. J. Microbiol. **45:**905–913.
64. **Tenover, F. C., and C. L. Fennell.** 1991. The genera *Campylobacter* and *Helicobacter*, p. 3488–3511. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed., vol. IV. Springer-Verlag, New York, N.Y.
65. **Timmer-Ten Hoor, A.** 1975. A new type of thiosulphate oxidizing, nitrate reducing microorganism: *Thiomicrospira denitrificans* sp. nov. Neth. J. Sea Res. **9:**344–350.
66. **Timmer-Ten Hoor, A.** 1981. Cell yield and bioenergetics of *Thiomicrospira denitrificans* compared with *Thiobacillus denitrificans*. Antonie Leeuwenhoek **47:**231–243.
67. **Van den Emde, F. P., and H. van Gemerden.** 1993. Sulfide oxidation under oxygen limitation: a *Thiobacillus thioparus* isolated from a marine microbial mat. FEMS Microbiol. Ecol. **13:**69–78.
68. **Vandamme, P., E. Falsen, R. Rossau, B. Hoste, P. Segers, R. Tytgat, and J. De Ley.** 1991. Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* taxonomy: emendation of generic descriptions and proposal of *Arcobacter* gen. nov. *Int. J. syst. Bacteriol.* **41:**88–103.
69. **Visser, J. M., L. A. Robertson, H. W. Van Verseveld, and J. G. Kuenen.** 1997. Sulfur production by obligately chemolithoautotrophic *Thiobacillus* species. Appl. Environ. Microbiol. **63:**2300–2305.
70. **Voordouw, G., S. M. Armstrong, M. F. Reimer, B. Fouts, A. J. Telang, Y. Shen, and D. Gevertz.** 1996. Characterization of 16S rRNA genes from oil field microbial communities indicates the presence of a variety of sulfate-reducing, fermentative, and sulfide-oxidizing bacteria. Appl. Environ. Microbiol. **62:**1623–1629.
71. **Vorholt, J. A., D. Hafenbradl, K. O. Stetter, and R. K. Thauer.** 1997. Pathways of autotrophic CO_2 fixation and of dissimilatory nitrate reduction to N_2O in *Ferroglobus placidus*. Arch. Microbiol. **167:**19–23.
72. **Widdel, F., and N. Pfennig.** 1991. The genus *Desulfuromonas* and other Gram-negative sulfur-reducing eubacteria, p. 3379–3389. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed., vol. IV. Springer-Verlag, New York, N.Y.
73. **Wolfe, R. S., and N. Pfennig.** 1977. Reduction of sulfur by *Spirillum* 5175 and syntrophism with *Clorobium*. Appl. Environ. Microbiol. **33:**427–433.
74. **Zumft, W. G.** 1991. The denitrifying prokaryotes, p. 554–582. *In* A. Balows, H. G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, 2nd ed., vol. I. Springer-Verlag, New York, N.Y.