

Genomic Organization of the Acidophilic Chemolithoautotrophic Bacterium *Thiobacillus ferrooxidans* ATCC 21834

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The genomic organization of the acidophilic chemolithoautotrophic bacterium *Thiobacillus ferrooxidans* ATCC 21834 has been studied by pulsed-field gel electrophoresis (PFGE). Analysis of its intact DNA, as well as the restriction patterns obtained with several endonucleases, allowed the characterization of one circular chromosome of 2.9 Mb and one plasmid of 8.6 kb. The first complete and highly resolved physical map (86 restriction sites) of the chromosome of an acidophilic obligate chemolithoautotrophic bacterium has been constructed by using endonucleases *PmeI*, *SwaI*, *XbaI*, and *SpeI*. The rRNA and *str* operons have been located on the chromosomal physical map.

Thiobacillus ferrooxidans is a gram-negative, acidophilic, chemolithotrophic bacterium that can fix atmospheric carbon dioxide, obtaining its energy through the oxidation of ferrous iron (7) as well as from the reduction of inorganic sulfur compounds (8). Some strains are also able to oxidize molecular hydrogen (4) and formic acid (13). *T. ferrooxidans* grows at extremely low pH values, as low as 1.5, and exhibits resistance to relatively high concentrations of heavy-metal ions (14). It has been described as one of the most important microorganisms involved in metal solubilization in mining operations (19) and coal desulfurization (2). However, its low growth rate limits its biotechnological applications. In spite of the interest in *T. ferrooxidans* as a potentially useful microorganism for biomining and as a model for the study of the chemolithoautotrophic way of life, very little progress has been made in the study of its genetics, mainly due to the lack of reliable techniques (for a recent review, see Rawlings and Kusano [14]).

Pulsed-field gel electrophoresis (PFGE) techniques provide genomic information about microorganisms to which conventional genetic techniques either cannot be applied or are difficult to implement. Using PFGE we have obtained genomic information on the acidophilic strict chemolithotroph *T. ferrooxidans* ATCC 21834 regarding the number, size, and topology of its genomic elements. Using a top-down approach we have constructed a complete restriction map of the chromosome for nucleases *PmeI*, *SwaI*, *XbaI*, and *SpeI*, on which several genes have been located.

MATERIALS AND METHODS

Growth of *T. ferrooxidans*. *T. ferrooxidans* ATCC 21834 (provided by the Kluyver Laboratory of Biotechnology, Delft University, as LMD 81.69) was chosen as the experimental strain. Bacteria were grown on a modified 9K medium (5), used as basal medium, supplemented with 30 mM thiosulfate, in a chemostat applying a dilution rate of 0.01 h^{-1} at 30°C and pH 3. The chemostat was aerated with air containing 2% CO₂, and agitation was provided by stirring at 700 rpm.

The organism was also grown in 0.2% elemental sulfur and molecular hydrogen in flasks shaken at 100 rpm. For cultures grown in molecular hydrogen, cells were taken from the chemostat and grown in anaerobic jars with a H₂-CO₂-air mixture (80:20:3). Cells were collected at an optical density of 0.7 at 430 nm and washed with substrate-free medium.

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Preparation of intact genomic DNA. DNA from bacterial cells grown with reduced sulfur compounds or molecular hydrogen was prepared by following a method described previously (16). DNA from bacteria grown with ferrous sulfate was obtained by following the protocol described in (9). All prepared blocks were stored in 0.5 M EDTA at 4°C.

Endonuclease restriction. Before restriction the agarose blocks were extensively washed in TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 8). Endonucleases were purchased from New England Biolabs and Boehringer Mannheim. Slices of blocks containing genomic DNA (1.5 µg) were included in the recommended restriction buffer containing 0.1 mg of acetylated bovine serum albumin per ml and the chosen enzyme (about 20 U) to a final volume of 100 µl. Incubations were carried out for 3 h at the appropriate temperature.

PFGE. Contour-clamped homogeneous electric field electrophoresis (CHEF) (3) was performed in a Pharmacia-LKB apparatus. Orthogonal-field alternating gel electrophoresis (OFAGE) (16) was carried out in a homemade apparatus consisting of a buffer chamber of 55 by 55 cm with four electrodes, following the prototype model described by Mathew et al. (11), plus an integrated pulser/power supply made by the technical service of the Centro de Biología Molecular C.B.M., as well as in a Pharmacia-LKB Pulsaphor system. Agarose gels (1%) were run in 0.5× TBE buffer (45 mM Tris-borate, 45 mM boric acid, 1 mM EDTA, pH 8) at 13°C. Different resolution windows were obtained by varying the pulse time between 1 and 2,000 s. Lambda DNA concatamers (Pharmacia), lambda phage CHEF DNA size standard 8 to 48 kb (Biorad), and chromosomes of *Saccharomyces cerevisiae* YN295, *Schizosaccharomyces pombe* 972 h⁻, and *Hansenula wingei* (Biorad) were used as linear DNA standards.

Southern hybridization and DNA labelling. DNA was transferred onto Hybond-N membranes (Amersham) by following the method described by Smith et al. (17). DNA fragments were digoxigenin labelled directly in agarose by random primed synthesis according to the procedure recommended by Boehringer Mannheim (DIG-System; Boehringer Mannheim). Hybridizations were carried out under the conditions described previously (10) overnight in high-stringency conditions (68 to 70°C). Blots were stored at 20°C and reused up to six times. Probe solutions were also maintained at -20°C and reused after denaturation (10 to 15 min at 90 to 100°C). Clones containing the rRNA and the *str* operons from *Thiobacillus cuprinus* were provided by D. Moreira (12).

RESULTS AND DISCUSSION

Genome organization, chromosome size, and topology. Given the absence of suitable techniques for genetic manipulation of the chemolithoautotrophic bacterium *T. ferrooxidans*, considerable interest has arisen in the use of PFGE techniques to acquire information on the genomic organization and the genetics of this microorganism. Several type collection strains were used to evaluate the possibility of preparing intact DNA and macrorestriction patterns suitable for PFGE analysis. Of those, *T. ferrooxidans* ATCC 21834 was selected due to its adequate growth in different chemolithotrophic substrates such as ferrous ion, elemental sulfur, thiosulfate, formic acid, and molecular hydrogen, which would facilitate the identifica-

TABLE 1. Lengths of *SpeI*, *XbaI*, *SwaI*, *PmeI*, and *SwaI-PmeI* restriction fragments of *T. ferrooxidans* ATCC 21834 (LMD 81.69) chromosomal DNA and derived total genome length^a

Restriction fragment	Size (kbp)	Restriction fragment	Size (kbp)	Restriction fragment	Size (kbp)
<i>SpeI</i>		<i>XbaI</i>		<i>SwaI</i>	
Sp1.....	225	Sp33.....	8.5	X28.....	12
Sp2.....	185	Sp34.....	7	X29.....	9.5
Sp3.....	155	Sp35.....	6.5	X30.....	5
Sp4.....	144	Total.....	2,887 ± 50	Total.....	2,890 ± 50
Sp5a, -b ^b	139				
Sp6.....	130	X1.....	290	<i>SwaI</i>	
Sp7.....	118	X2.....	275	S1.....	850
Sp8.....	115	X3.....	192	S2.....	475
Sp9.....	95	X4.....	188	S3.....	425
Sp10.....	92	X5.....	185	S4a, -b ^b	385
Sp11a, -b ^b	90	X6.....	167	S5.....	330
Sp12.....	83	X7.....	142	Total.....	2,850 ± 50
Sp13.....	80	X8a, -b ^b	128	<i>PmeI</i>	
Sp14.....	73	X9.....	100	P1.....	2,200
Sp15.....	70	X10.....	92	P2.....	500
Sp16a, -b ^b	65	X11.....	90	P3.....	175
Sp17.....	60	X12a, -b ^b	77	P4.....	25
Sp18.....	55	X13a, -b ^a	73	Total.....	2,900 ± 50
Sp19a, -b ^b	52	X14.....	68	<i>SwaI-PmeI</i>	
Sp20.....	48	X15a, -b ^b	53	SP1.....	498
Sp21a, -b ^b	47	X16.....	51	S2.....	475
Sp22a, -b ^b	46	X17.....	48	S3.....	425
Sp23.....	44	X18.....	46	S4a.....	385
Sp24.....	39	X19.....	34	SP2.....	383
Sp25.....	36	X20.....	31	S5.....	330
SP26.....	34	X21.....	30	P3.....	175
Sp27a, -b ^b	23	X22.....	25	SP3.....	150
Sp28.....	20	X23.....	23	P4.....	25
Sp29a, -b ^b , -c ^b	17	X24.....	21	SP4.....	1.8
Sp30.....	16	X25a, -b ^b	19	Total.....	2,848 ± 50
Sp31.....	10	X26a, -b ^b	18		
Sp32.....	9	X27a, -b ^b	15		

^a Each restriction band is named by the initial letter of the enzyme followed by the order number in the digestion pattern. Total size was calculated as an average value in kilobases from two to four size determinations.

^b Comigrating fragments.

tion of genes involved in chemolithotrophy by comparative gene expression at different growth conditions.

It is well established that circular DNAs greater than 1 Mb remain in the well during PFGE, while linear forms, up to 10 Mb, can be separated by this technique using long pulse times, long runs, and low electric fields (17). Figure 1 shows that after electrophoresis of intact total DNA from *T. ferrooxidans* ATCC 21834, most of the DNA remains trapped in the agarose blocks (lane 4). The randomly linearized form of the chromosome can be observed with a mobility, 2.9 Mb, similar to that of the linearized chromosomal band corresponding to partial digestions with *PacI* (lane 3) and *PmeI* (lanes B1). These results strongly suggest that *T. ferrooxidans* ATCC 21834 has a circular chromosome of 2.9 Mb.

Extrachromosomal elements. The presence of a low-copy-number plasmid was detected when intact DNA from cells grown under different chemolithotrophic conditions were analyzed at low pulse times. The plasmid band was isolated, labelled, and used as a hybridization probe. The detection by hybridization of two DNA species suggested that they corresponded to different topological forms of the same molecule. It has been shown that in PFGE small covalently closed circular DNAs (cccDNAs), open circular and supercoiled molecules of less than 16 kb (6) migrate in a pulse-time-independent manner compared to linear markers (1, 10). To further characterize the possible different topological forms of the plasmid, we

carried out Southern hybridizations using plasmid DNA probe against PFGE blots containing *T. ferrooxidans* genomic DNA previously treated with *AseI*, *SpeI*, and *XbaI*. Digestion with *AseI* linearizes the plasmid, allowing its size to be estimated as 8.6 kb (Fig. 2B), while *SpeI* and *XbaI* cleavage sites were not

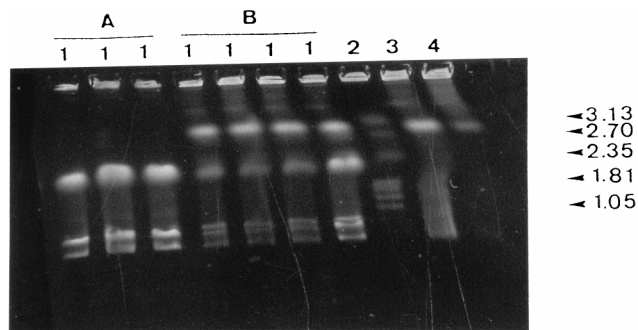


FIG. 1. PFGE analysis of chromosomal DNA of *T. ferrooxidans* ATCC 21834. Fragments were separated by using a 200-V, 2,000-s pulse time for 5 days. Samples were total *PmeI* digestion patterns (lanes A1) and partial *PmeI* (lanes B1) and *PacI* (lane 3) digestion fragments showing a 2.9-Mb band corresponding to the linear chromosome and intact total DNA showing random linearized chromosomal DNA (lane 4). Chromosomes of *H. wingei* (ranging from 3.13 to 1.05 Mb in size) were used as size markers (lane 2).

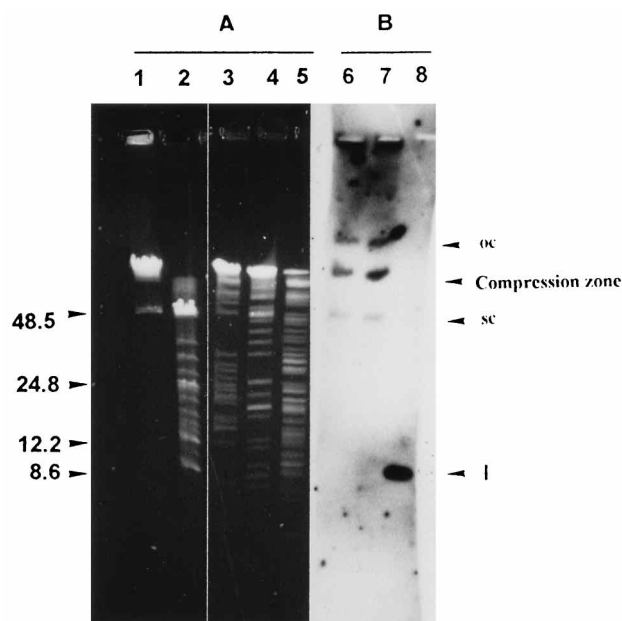


FIG. 2. Characterization of the plasmid DNA from *T. ferrooxidans* by restriction fragment analysis. CHEF running conditions were 2-s pulse time, 10 V cm^{-1} for 24 h. (A) *T. ferrooxidans* DNA *Xba*I restriction fragments (lane 3), *Spe*I restriction fragments (lane 4), and *Ase*I restriction fragments (lane 5). (B) Correspondent hybridization with plasmid DNA (lanes 6 to 8). Lambda concatamers (lane 1) and lambda phage size standard from Biorad (lane 2) were used as markers. Size values are given in kilobase pairs.

found. However, the incubation of total DNA in the restriction buffer together with the electric field effect during the electrophoresis produces single-strand cuts, generating circular-relaxed forms. By this method three plasmid forms, supercoiled (sc), open circular (oc), and linear (l), were detected by Southern hybridization (Fig. 2B).

Restriction patterns. To facilitate the obtention of the chromosomal physical map, different endonucleases were tested in order to select the most appropriate ones. The best results were obtained with *Swa*I (six fragments) and *Pme*I (four fragments). Two other endonucleases, *Xba*I and *Spe*I, generate a larger number of fragments, 37 and 44 fragments, respectively, the resolution of which required the use of different running conditions. Table 1 shows the different restriction fragments obtained with the endonucleases used in this work. The fragments are named alphanumerically from the largest to the smallest. The chromosome size calculated for each enzyme, including *Pme*I-*Swa*I double digestions, ranged from 2,850 to 2,930 kb, which corroborates the chromosomal size previously estimated by resolution of the linearized chromosome (Fig. 1).

Physical map of the chromosome. The following methods were combined to organize the restriction fragments into a physical map: (i) partial digestion with a given enzyme, (ii) combination of single and double digestions, and (iii) hybridization with restriction fragments and gene probes. Once the chromosomal restriction patterns for *Swa*I and *Pme*I were established, the next step was to identify their relative position in order to generate a low-resolution physical map. A first approximation was based on complete and partial digests with *Swa*I and *Pme*I. On one hand, *Swa*I fragments were labelled and hybridized to Southern blots of *Swa*I partial genomic digests fractionated by PFGE. On the other hand, *Pme*I partial digests allowed the four *Pme*I fragments to be aligned linearly (Fig. 3). We also performed simple *Swa*I and *Pme*I digestions

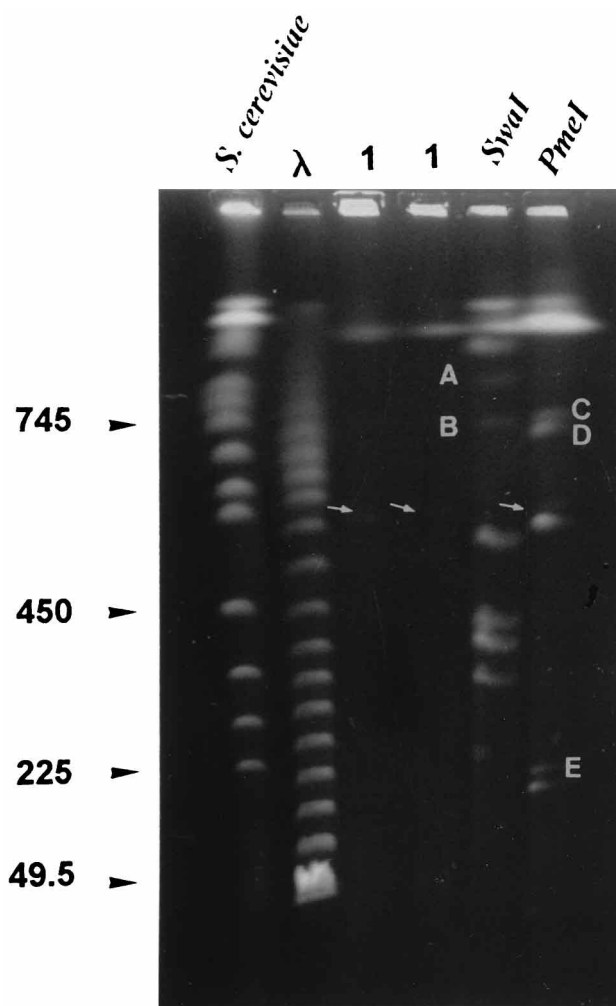


FIG. 3. PFGE (CHEF) separation of *Swa*I and *Pme*I partial restriction fragments of *T. ferrooxidans*. In *Swa*I digestions there are two partially digested bands: S1.1 fragment of 810 kb (A) and S1.2 fragment of 715 kb (B). In *Pme*I digestions there are three partially digested bands: P1.1 of 700 kb, corresponding to the sum of P2, P3, and P4 fragments (C), P1.2 of 675 kb, corresponding to the sum of P2 and P3 fragments (D), and P2.1 of 200 kb, corresponding to the sum of P3 and P4 fragments (E). Lane 1 corresponds to intact genomic DNA from *T. ferrooxidans*. White arrows show the bands corresponding to the plasmid. Lambda DNA concatamers and *S. cerevisiae* chromosomes were used as size standards, and their sizes, in kilobase pairs, are indicated on the left. Running conditions were 200 V for 26 h. Pulse time was 40 s for 13 h, followed by pulses of 70 s for 13 h.

and *Swa*I-*Pme*I double digestions. Four new DNA fragments, named SP1 to SP4, were generated by double digestions with *Swa*I-*Pme*I (SP3, 150 kb; SP4, 1.8 kb). SP3 corresponds to the overlapping zone between S1 and P1, and SP4 corresponds to the overlapping zone between P2 and S4b fragments. After double digestion these two bands disappeared and two new ones appeared, SP1 and SP2 (Table 1). The small difference in size between these bands and those from which they originate (P2 and S4b) explains why they migrate with similar mobilities. Complementary information was obtained by hybridization experiments. By combining all these data, a low-resolution physical map for *Swa*I and *Pme*I was constructed.

By using this map as reference, the recognition sites for two additional enzymes, *Spe*I and *Xba*I, were located by hybridization. The high-resolution macrorestriction map of *T. ferroxi-*

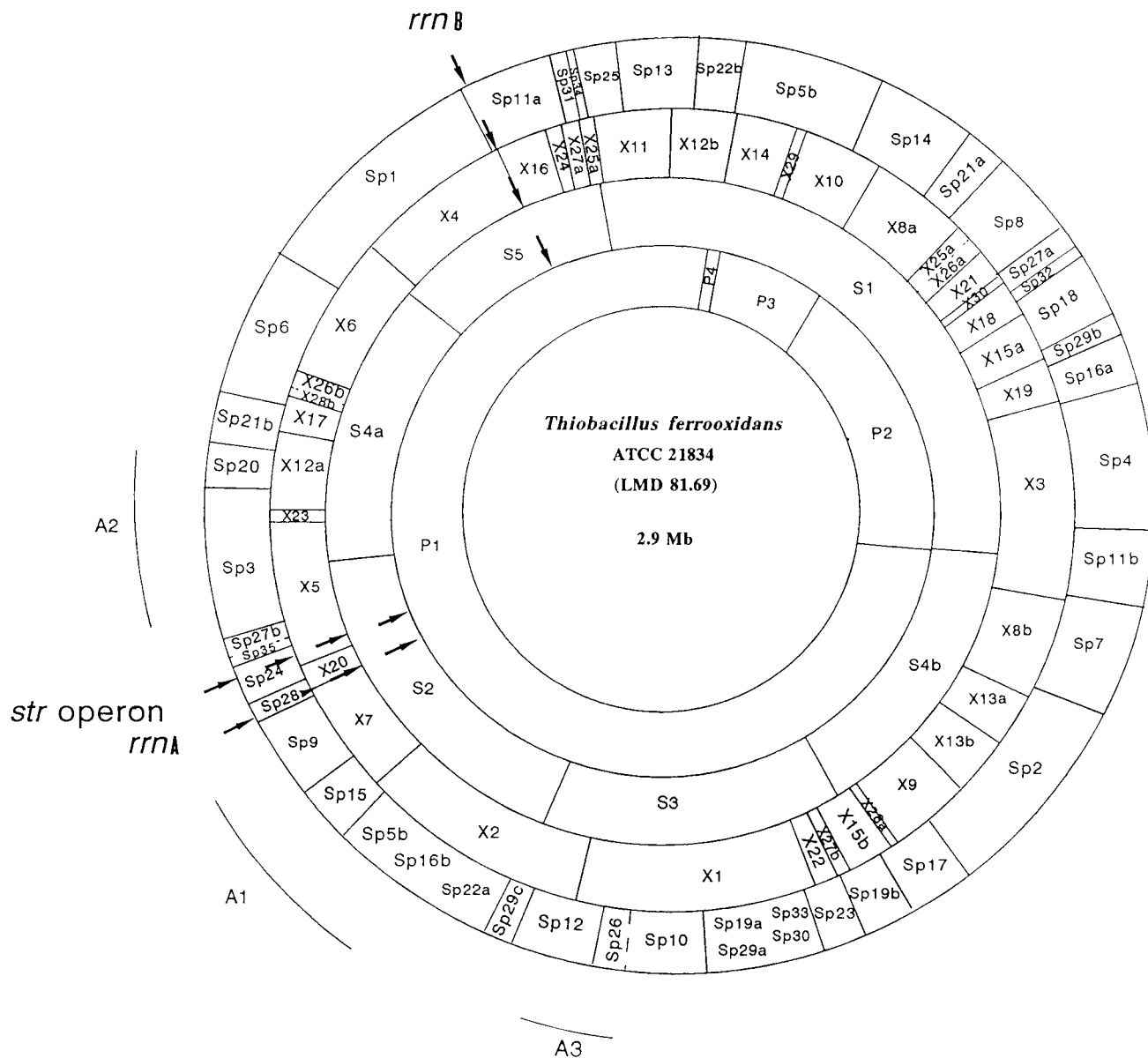


FIG. 4. Physical map of *T. ferrooxidans* ATCC 21834 (LMD 81.69) chromosome. *PmeI* (P), *SwaI* (S), *XbaI* (X), and *SpeI* (Sp) fragments are indicated by letters from the inner to the outer circle. The relative orders of X25a and X26a and of X28b and X26b are tentative. Outside the circle, several fragments generated by *AseI* (A) are indicated. The locations of rRNA and *str* operons in the chromosomal physical map are indicated.

dans ATCC 21834, obtained from these diverse approaches, is shown in Fig. 4. The 4 *PmeI* fragments, the 6 *SwaI* fragments, and the 37 *XbaI* fragments form a circle, corroborating the circular topology of the *T. ferrooxidans* chromosome.

Gene location. One way to study the genetic organization of *T. ferrooxidans* is to locate genes on the physical map in order to generate a genetic map. Two heterologous hybridization probes, the rRNA and the *str* operons from *T. cuprinus*, were used to locate the homologous genes on the restriction map of *T. ferrooxidans* chromosome. Two rRNA operons have been detected in the *T. ferrooxidans* ATCC 21834 chromosome, which agreed with the data previously reported by Salazar et al. when a related strain was used (15). The hybridization patterns suggest that one of the rRNA operons is closely linked to the *str* operon, as found in other thiobacilli (12).

The description of the genomic structure of *T. ferrooxidans*

reported here constitutes a foundation for further studies. The identification of genes on this physical map can be used to establish correlations between gene structure and function, which may facilitate the understanding of chemolithoautotrophy and its biotechnological application.

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REFERENCES

1. **Beverley, S. M.** 1988. Characterization of the 'unusual' mobility of large circular DNAs in pulse field-gradient electrophoresis. *Nucleic Acids Res.* **16**:925-939.
2. **Bos, P., F. C. Boogerd, and J. G. Kuenen.** 1992. Microbial desulfurization of coal, p. 375-403. *In* Ralph Mitchell (ed.), *Environmental microbiology*. Wiley-Liss, Inc., New York, N.Y.
3. **Chu, G., D. Vollrath, and R. W. Davies.** 1984. Separation of large DNA molecules by contour-clamped homogenous electric fields. *Science* **234**:1582-1585.
4. **Drobner, E., H. Huber, and K. O. Stetter.** 1990. *Thiobacillus ferrooxidans*, a facultative hydrogen oxidizer. *Appl. Environ. Microbiol.* **56**:2922-2923.
5. **Hazeu, W., W. Bijleveld, J. T. C. Grotenhuis, E. Kakes, and J. G. Kuenen.** 1986. Kinetics and energetics of reduced sulfur oxidation by chemostat cultures of *Thiobacillus ferrooxidans*. *Antonie van Leeuwenhoek* **52**:507-518.
6. **Hightower, R. C., D. W. Metge, and D. V. Santi.** 1987. Plasmid migration using orthogonal-field-alternation gel electrophoresis. *Nucleic Acids Res.* **15**:8387-8398.
7. **Ingledeu, W. J.** 1982. *Thiobacillus ferrooxidans*: the bioenergetics of an acidophilic chemolithotroph. *Biochem. Biophys. Acta* **683**:89-117.
8. **Kelly, D. P., and A. P. Harrison.** 1989. Genus *Thiobacillus*, p. 1842-1858. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 3. The Williams & Wilkins Co., Baltimore, Md.
9. **Kondrat'eva, T. F., and G. I. Karavaiko.** 1992. Restriction analysis of *Thiobacillus ferrooxidans* DNA by electrophoresis in pulsating differently-directed electric fields. *Molekul. Gen. Mikrobiol. Virusol.* **3**:9-12.
10. **López-García, P., J. Antón, J. P. Abad, and R. Amils.** 1994. Halobacterial megaplasmids are negatively supercoiled. *Mol. Microbiol.* **11**:421-427.
11. **Mathew, M. K., C. L. Smith, and C. R. Cantor.** 1988. High resolution separation and accurate size determination in pulsed-field gel electrophoresis of DNA. II. Effect of pulse time and electric field strength and implications for models of the separation process. *Biochemistry* **27**:9210-9216.
12. **Moreira, D.** 1995. Ph.D. thesis. Universidad Autónoma de Madrid, Madrid, Spain.
13. **Pronk, J. T., W. M. Meijer, W. Hazeu, J. P. van Dijken, P. Bos, and J. G. Kuenen.** 1991. Growth of *Thiobacillus ferrooxidans* on formic acid. *Appl. Environ. Microbiol.* **57**:2057-2062.
14. **Rawlings, D. E., and T. Kusano.** 1994. Molecular genetics of *Thiobacillus ferrooxidans*. *Microbiol. Rev.* **58**:39-55.
15. **Salazar, O., M. Takamiya, and O. Orellana.** 1989. Characterization of the two rRNA gene operons present in *Thiobacillus ferrooxidans*. *FEBS Lett.* **242**:439-443.
16. **Schwartz, D. C., and R. Cantor.** 1984. Separation of yeast chromosome-sized DNAs by pulsed field gel electrophoresis. *Cell* **37**:67-75.
17. **Smith, C. L., and G. Condemine.** 1990. New approaches for physical mapping of small genomes. *J. Bacteriol.* **172**:1167-1172.
18. **Smith, C. L., S. R. Klcó, and C. R. Cantor.** 1988. Pulsed field gel electrophoresis and the technology of large DNA molecules, p. 41-72. *In* K. Davies (ed.), *Genome analysis: a practical approach*. IRL Press, Oxford, England.
19. **Torma, A. E.** 1988. Leaching of metals, p. 367-399. *In* H. J. Rehm and G. Reed (ed.), *Biotechnology*, vol. 6B. VCH Verlagsgesellschaft, Weinheim, Germany.