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Molecular Genetics of *Thiobacillus ferrooxidans*

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INTRODUCTION

Considerable interest has been shown in *Thiobacillus ferrooxidans* because of its use in industrial mineral processing and its unusual physiology. The major contribution of *T. ferrooxidans* to metal extraction is its ability to attack sulfide-containing minerals and convert the insoluble sulfides of metals such as copper, lead, zinc, or nickel to their soluble metal sulfates. The three metals recovered in the largest quantities through direct or indirect bacterial action are copper (15), uranium (88), and, more recently, gold (83).

Industrial bioleaching processes are not carried out under sterile conditions, and as a result *T. ferrooxidans* seldom if ever grows as a pure culture. Most commercial bioleaching operations are carried out with a consortium of highly acidophilic, chemolithotrophic bacteria which may include *T. ferrooxidans*, *Thiobacillus thiooxidans*, *Leptospirillum ferrooxidans*, and acidophilic heterotrophic bacteria belonging to the genus *Acidiphilium* (41, 63). Although *T. ferrooxidans* is considered to be the most important member of the consortium, mixed cultures of bacteria are often more efficient at ore decomposition than is *T. ferrooxidans* alone. The above bacteria are all mesophilic, and as a result, when control is possible the industrial biooxidation of ores is carried out at temperatures below 45°C.

At temperatures greater than 45°C, several other types of bacteria including the moderately thermophilic "*Thiobacillus* TH" strains and various *Sulfobacillus* species dominate (92). At even higher temperatures, extremely thermophilic archaebacteria such as *Sulfolobus* and *Acidianus* species are found. These groups of bacteria are also able to rapidly attack a variety of ores. However, there are currently no industrial processes that deliberately use these thermophilic bacteria.

The biooxidation reactions and proposed mechanisms for the bioleaching of a number of minerals have already been reviewed and will not be covered again here (15, 65, 84).

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Likewise, numerous popular and scientific articles on the industrial use of *T. ferrooxidans*-dominated cultures have been published (16, 89).

PHYSIOLOGY OF *T. FERROOXIDANS*

T. ferrooxidans is a gram-negative, rod-shaped bacterium that has a physiology which is well suited for growth in an inorganic mining environment. It obtains its carbon by fixing atmospheric carbon dioxide and is obligately autotrophic (64). A number of early reports on its ability to grow heterotrophically are considered to be erroneous. *T. ferrooxidans* cultures are notoriously difficult to free from associated heterotrophic bacteria belonging to the genus *Acidiphilium* and it is the growth of these bacteria that was mistaken for growth of *T. ferrooxidans* (41). One strain has been reported to grow mixotrophically in the presence of iron and glucose (8) but in general, organic compounds inhibit growth (2, 143). Formic acid can replace carbon dioxide as the carbon source in most, but not all, *T. ferrooxidans* strains examined (100). However, the concentration of formate must be kept low through the use of a chemostat. One strain, ATCC 21834, was particularly efficient at consuming formic acid.

Not only is the bacterium autotrophic, but also it is capable of fixing atmospheric nitrogen (85). All 15 *T. ferrooxidans* strains examined have the genes for nitrogen fixation (98, 105). Diazotrophy is therefore likely to be a general property of these bacteria.

Energy is obtained by the oxidation of either ferrous ions to ferric or reduced-sulfur compounds to sulfuric acid. The bacterium is acidophilic with an optimum pH within the range pH 1.5 to 2.5. It grows best in an aerobic environment with oxygen as an electron acceptor. If oxygen is lacking and reduced-sulfur compounds or formate is used as the electron donor, ferric ions can serve as the electron acceptor (22, 99, 136, 137). Other compounds besides iron and sulfur may be oxidized. Three *T. ferrooxidans* strains, including the type strain ATCC 23270, grow in a basal-salts medium when incubated in the presence of CO_2 and O_2 and with H_2 as the sole energy source (31). There are also reports that *T. ferrooxidans* can grow via the direct oxidation of UO_2 in the presence of iron (27) and that it possesses a molybdenum-oxidizing enzyme (135).

Most isolates of *T. ferrooxidans* have remarkably modest nutritional requirements. Aeration of a sample of iron pyrite in acidified water is sufficient to support growth at the expense of the pyrite. The pyrite provides the energy source and trace elements; the air provides the carbon, nitrogen, and oxygen; and the acidified water provides the growth environment. *T. ferrooxidans* is ubiquitous in the environment. It may be readily isolated from soil samples collected from the vicinity of pyritic ore deposits or from sites of acid mine drainage that are frequently associated with coal waste or mine dumps.

In addition to its unique physiology, *T. ferrooxidans* has other features that make it suitable for use in biomining operations. One of these is its inherent resistance to high concentrations of metallic and other ions (142). For example, the bacterium has been reported to be capable of growth in medium containing Zn^{2+} (120 g/liter), Ni^{2+} (72 g/liter), Co^{2+} (30 g/liter), Cu^{2+} (55 g/liter), U_3O_8 (12 g/liter), and Fe^{2+} (160 g/liter) (140). Another feature is the remarkable adaptability of the organism when faced with adverse growth conditions. Previously sensitive strains have been adapted to high concentrations of arsenic (116) and copper (45), lower-than-optimum pH (148), and a number of other factors. The mechanism of adaptation is possibly due to the accumulation of advantageous mutations under selective conditions, but this has not been established. Schrader and Holmes have suggested (127) that there may also be other mechanisms which contribute to the rapid adaptation of the bacterium to harsh conditions (see the section on repeated sequences and phenotypic switching).

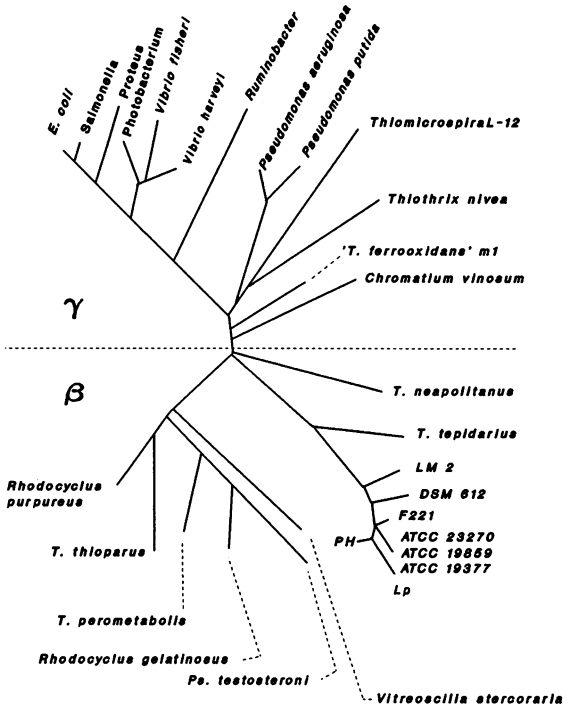

FIG. 1. The 16S rRNA relationships of several *Thiobacillus* isolates within the β and γ subdivisions of the *Proteobacteria*. LM2, F221, ATCC 23270 (type strain), ATCC 19859, Lp, and PH are classified as strains of *T. ferrooxidans*, while DSM 612 and ATCC 19377 (type strain) are strains of *T. thiooxidans*. Redrawn from reference 78 with permission of the publisher.

Not all isolates of bacteria that are referred to as *T. ferrooxidans* are genetically closely related. Harrison (41) carried out a systematic study of a large number of *T. ferrooxidans* strains and reported that the G+C content of their DNA could vary from 55 to 65 mol%. On the basis of interstrain hybridization studies, he divided *T. ferrooxidans* strains into seven different DNA homology groups. In later studies, 5S (79) and partial 16S (78) rRNA sequences were used to phylogenetically compare strains of *T. ferrooxidans* with each other and other acidophiles (Fig. 1). On the basis of the 16S rRNA data, most *T. ferrooxidans* strains, including representatives from three of Harrison's homology groups, were placed into a separate phylogenetic cluster. An exception was the *T. ferrooxidans* strain m-1, which was clearly different from the main grouping. When the phylogenetic relationship of *T. ferrooxidans* strains with other gram-negative bacteria was investigated, the main cluster fitted best into the β subdivision (152) of the *Proteobacteria*. In contrast, strain m-1 appeared to fall within the *Proteobacteria* γ subdivision. It is interesting that the two *T. thiooxidans* strains examined in the 16S rRNA study, including the type strain ATCC 19377, were grouped together with the main *T. ferrooxidans* cluster (78). There is clearly sufficient variation among bacteria that carry the name *T. ferrooxidans*, for them to be subdivided into several species within the genus. The genus *Thiobacillus* also includes many neutrophilic, facultatively autotrophic bacteria which, because they have the

same genus designation, are frequently mistaken to be closely related to *T. ferrooxidans*. The 16S rRNA sequence data and physiological characteristics of these "neutrophilic thiobacilli" are so different from typical acidophilic, obligately autotrophic *T. ferrooxidans* and *T. thiooxidans* isolates that they should not be considered to be members of the same genus.

Why Study the Molecular Genetics of *T. ferrooxidans*?

In spite of its suitability for growth in an inorganic mining environment, *T. ferrooxidans* does have limitations. These are not readily apparent in low-rate operations such as the leaching of ore dumps or heaps but become more obvious in high-rate processes such as the vat leaching of gold-bearing arsenopyrite concentrates (146). One of the most noticeable limitations is the slow doubling time of the bacterium. Since the organisms do not settle (like a sewage sludge), there is no ready means of recycling and the minimum retention time in a leaching vat is restricted by the low growth rate of the bacteria. An empirical approach to solving this problem is through the optimization of fermentation conditions such as the rate of aeration, the concentration of CO_2 and O_2 in the air, temperature, pH, and the addition of nutrients such as NH_4^+ and PO_4^{3-}. A more fundamental approach is to increase our understanding of the nutrition and energy generation mechanisms of the bacterium's highly demanding chemoautolithotrophic way of life. This approach includes the development of the genetic systems needed to carry out some of the physiological studies and to genetically manipulate commercial strains if required. Most studies on the molecular genetics of *T. ferrooxidans* have been carried out with these objectives in mind.

NATURAL PLASMIDS FROM *T. FERROOXIDANS*

A number of surveys of the occurrence of natural plasmids in strains of *T. ferrooxidans* isolated from different parts of the world have been made. Martin et al. (86) reported that 11 of 15 strains originating mostly from the United States and Bulgaria contained one to five plasmids per strain ranging in size from 7.4 to 75 kbp. In a survey of more than 100 strains from six Japanese mining sites, 73% were found to carry one or more plasmids ranging in size from 2.0 to 30 kbp (131). Plasmids have also been reported in strains from South Africa (109), Italy and Mexico (145), Chile (124), and Canada (87). In the Canadian study, one to four plasmids of 18.6 to 65 kbp were detected in nine *T. ferrooxidans* strains isolated from the Agnew Lake uranium mine, northern Ontario. An attempt to correlate the presence of a plasmid of a particular size with an increase in uranium resistance was made. These workers found that the four strains which exhibited the highest resistance to UO_2^{2+} all contained a particular 20-kbp plasmid. In one strain the disappearance of the 20-kbp plasmid occurred together with a reduction in uranium resistance (87). However, this evidence is circumstantial and insufficient to prove a causal relationship. Other attempts have been made to correlate the presence of naturally occurring plasmids with metal resistance, but these have not been successful. Part of the problem is that without a selectable marker, curing strains of their natural plasmids is very difficult and no attempts at curing have been successful. Although the occurrence of plasmids is clearly widespread among strains of *T. ferrooxidans*, no phenotype has yet been ascribed to any *T. ferrooxidans* plasmid.

Several *T. ferrooxidans* plasmids have been cloned in *Escherichia coli*. These include two plasmids from the uranium-resistant ATCC 33020 strain (44); one from the arsenic-resistant FC strain (111); a 20-kbp plasmid that has been found in *T. ferrooxidans* strains isolated from many different parts of Italy, Sardinia, and Bulgaria (109, 145); and four plasmids from *T. ferrooxidans* strains isolated in Japan (131). In one study four recombinant *T. ferrooxidans* plasmids were transformed into *E. coli* and the resistance of the transformants to Ag^+, As^{3+}, As^{5+}, Cd^{2+}, Co^{2+}, Cr^{6+}, Cu^{2+}, Hg^+, Li^+, Mo^{6+}, Ni^{2+}, Sb^{3+}, Te^{4+}, U^{6+}, Zn^{2+}, and eight antibiotics was determined (113). The recombinant plasmids did not affect the tolerance of the transformants to the metal ions or confer resistance to any of the antibiotics.

Some functions of *T. ferrooxidans* plasmids have been expressed in *E. coli*. Rawlings et al. (111) reported that a plasmid isolated from *T. ferrooxidans* was capable of replication in *E. coli* and that three of four recombinant plasmids were mobilized between *E. coli* strains (115) by a coresident IncP plasmid, such as RP4. Two *T. ferrooxidans* plasmids have been studied in some detail.

Plasmid pTF1

Plasmid pTF1 is a 6.7-kb, mobilizable plasmid that was originally cloned into pBR322 from *T. ferrooxidans* ATCC 33020 by Holmes et al. (44). A 2.8-kbp region which is required for mobilization has been located and sequenced by Drolet et al. (33). Two proteins, the 42.6-kDa MobL and the 11.4-kDa MobS, that were essential for mobilization were identified and the exact position of the nick site within the oriT region was determined. The amino acid sequences of MobL and MobS were 49 and 53% similar to the MobA and MobC proteins of the broad-host-range IncQ plasmids, respectively. MobL was also related (45% amino acid sequence similarity) to the presumptive Mob protein of plasmid pSC101. An examination of the transcription and regulation of the *mobL* and *mobS* promoters in *E. coli* was carried out. Primer extension experiments indicated that the *mobL* and *mobS* genes were transcribed from a set of divergent σ^{70}-type promoters located within the oriT region (32). MobL but not MobS was found to specifically bind to a 42-bp fragment of single-stranded DNA which included a potential stem-loop structure and the oriT nick site.

A 1.3-kbp region of pTF1 which bears some resemblance to the replicon of pSC101 and which contains an open reading frame (ORF) whose predicted product has structural similarity to the DNA-binding KfrA protein of plasmid RK2 has been identified (80).

Plasmid pTF-FC2

Plasmid pTF-FC2 is a 12.2-kb, broad-host-range, highly mobilizable plasmid that was present in the arsenic-resistant *T. ferrooxidans* FC isolated in South Africa. This strain is a dominant member of a mixed bacterial culture that is used industrially in the biooxidation of a gold-bearing arsenopyrite concentrate (146). Plasmid pTF-FC2 is particularly interesting because few small broad-host-range plasmids are known. The entire pTF-FC2 plasmid has recently been sequenced and consists of three regions: a replicon region, a mobilization region, and a transposon-like element (108). The replication and mobilization regions have been studied in detail.

The replicon consists of five or six genes and an oriV region which is required in *cis* (Fig. 2). The oriV region has been located to a 184-bp fragment which includes three tandem perfectly repeated 22-bp sequences (29). This region is clearly related to the oriV of the broad-host-range IncQ plasmids (RSF1010, R1162, and R300B) which have 3.5 perfectly conserved 20-bp sequences separated by 2-bp nonconserved spacer sequences (82). If the two sets of repeated sequences

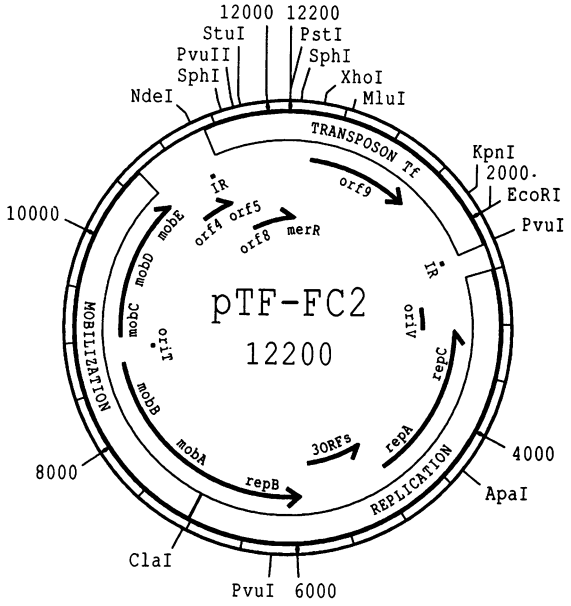

FIG. 2. Physical and genetic map of pTF-FC2, a broad-host-range, mobilizable plasmid isolated from *T. ferrooxidans*. Regions required for replication and mobilization, as well as a transposon-like element, are shown. Blocks labeled IR represent the inverted repeat sequences that border a transposon-like element and which have a 38-of-38 and 37-of-38 match to the IR sequences of *Tn*21. Modified from reference 108 with permission of the publisher.

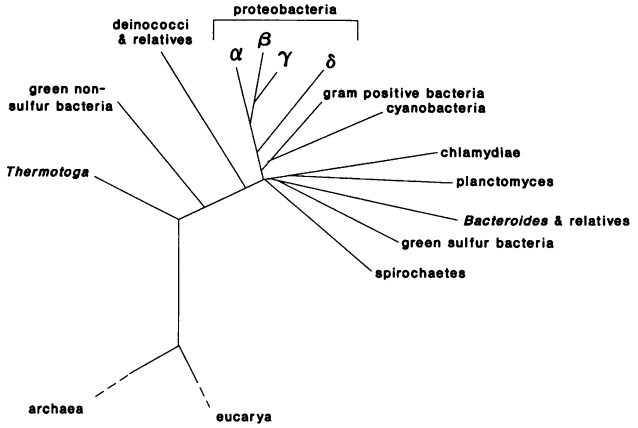

FIG. 3. Phylogenetic tree of the eubacteria based on 16S rRNA sequences. Branch lengths are proportional to evolutionary distances, with separate branches shown for the α, β, γ, and δ groups of proteobacteria. Redrawn from reference 17 with permission of the publisher.

including the spacers are aligned, there is 60% DNA homology between them. There is also a striking 75% DNA homology between the 115 bp of DNA immediately adjacent to the repeated sequences of pTF-FC2 and the IncQ plasmids (29). In the IncQ plasmid R1162, two distinct domains, contained on adjacent 370- and 210-bp *Hpa*II fragments are required in *cis* for plasmid replication (67). These two domains are able to direct plasmid replication even when the distance between them is increased. The extensive homology between the 184-bp oriV domain of pTF-FC2 and R1162 is contained entirely within the 370-bp *Hpa*II fragment. Plasmid pTF-FC2, therefore, does not appear to have a requirement for a domain equivalent to the 210-bp *Hpa*I fragment of the IncQ plasmids. The repeated sequences of the oriV have been shown to be responsible for incompatibility and copy number control in the IncQ plasmids (82) and incompatibility in pTF-FC2 (29). Although the repeated sequences of the two plasmids were 60% homologous, no incompatibility between the two types of plasmid was detected.

The similarity between the replicons of pTF-FC2 and the IncQ plasmids extends to three of the proteins required for replication. In the IncQ plasmids, the RepA protein functions as a helicase, RepB acts as an oriV specific primase, and RepC is a protein that binds specifically to the 20-bp directly repeated sequences of the oriV (40). When the Rep proteins from pTF-FC2 and the IncQ plasmid RSF1010 were aligned, there was 43, 26, and 60% amino acid sequence identity between the RepA, RepB, and RepC polypeptides, respectively (28, 30, 126). Although the percentage identity of the RepB proteins was lower than the other two, Dorrington et al. (28) showed that the pTF-FC2 RepB protein functioned as a primase which was required for the initiation of replication at the oriV of pTF-FC2. In spite of the relatedness between the Rep proteins of the two plasmids, an IncQ plasmid was unable to complement *repA*, *repB*, or *repC* mutants of pTF-FC2 (28, 30).

The extent of the host range of pTF-FC2 has not yet been tested beyond the gram-negative proteobacteria (previously called purple bacteria). These bacteria were divided into four groups, α, β, γ, and δ, on the basis of features of their 16S RNA sequences (Fig. 3) (152). Plasmid pTF-FC2 has been found to be capable of replication in at least one representative of each of the four groups: *Rhizobium meliloti*, *Acidiphilium facilis* (19), and *Agrobacterium tumefaciens* of the α group; *Thiobacillus novellus* and by implication *T. ferrooxidans* of the β group; *E. coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, and *Klebsiella pneumoniae* of the γ group; and *Myxococcus xanthus* of the δ group (34).

The mobilization region of pTF-FC2 is located on a 3.5-kb DNA fragment and consists of an oriT and five *mob* genes arranged in two operons on either side of the oriT (119). The two operons are divergently transcribed from two promoters located within the oriT region, *mobA* and *mobB* in one direction and *mobC*, *mobD*, and *mobE* in the other (Fig. 2). Three of the Mob proteins (MobA, MobC, and MobD) are essential for mobilization while the remaining two (MobB and MobE) affect mobilization frequency. An unexpected finding was that the mobilization region of pTF-FC2 was clearly although somewhat distantly related to the *Tra*I region of the broad-host-range conjugative IncP plasmids RP4 and R751 (160) (Fig. 4). Four of the Mob proteins had significant amino acid sequence homology (26 to 33% identity; 44 to 51% similarity) to four of the proteins of the *Tra*I region of the IncP plasmids. The approximate size and relative positioning of the proteins with respect to the oriT was also similar.

There are, however, some differences. The TraM polypeptide of RP4 has no detectable homology with the MobE protein of pTF-FC2. Plasmid RK2, which is very similar or identical to RP4, encodes a primase (*traG* gene product) which is required for specific priming at the oriT (153). In contrast, priming of replication at the pTF-FC2 oriT in *E. coli* did not require a plasmid-encoded primase (120), and this function is presumably provided by the host cell.

The third distinct region of pTF-FC2 was a transposon-like element (Fig. 2). This region was bordered by two identical 38-bp inverted repeat sequences which had a 37- of 38-bp and

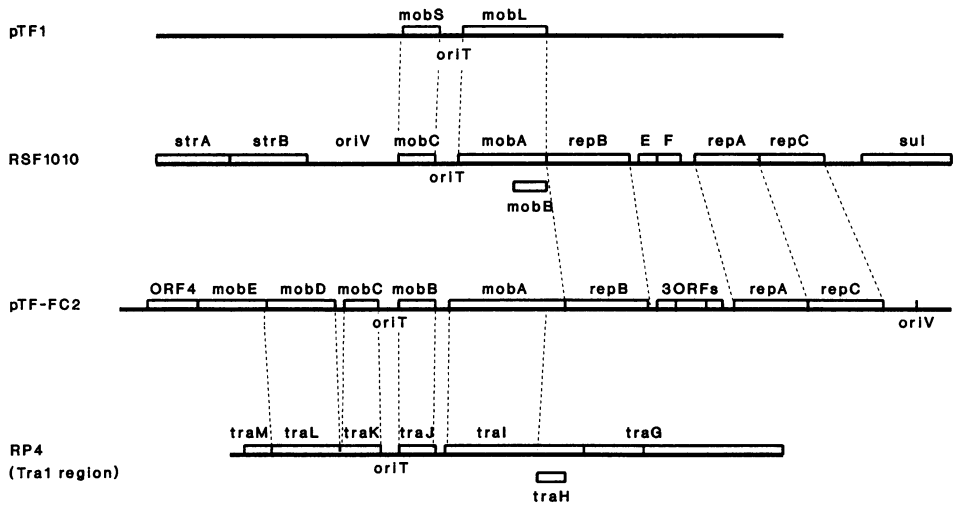

FIG. 4. Comparison between the mobilization and replication regions of four plasmids. Regions of significant sequence similarity are shown by broken parallel lines. The *mob* genes of the *T. ferrooxidans* plasmid pTF1 have clear similarity with the *mob* genes of RSF1010, a broad-host-range, mobilizable IncQ plasmid. The *rep* genes of the *T. ferrooxidans* plasmid pTF-FC2 have sequence similarity with the *rep* genes of RSF1010, whereas the mobilization region of pTF-FC2 is clearly related to the Tra1 region of the broad-host-range IncP plasmid RP4.

38- of 38-bp match to the left- and right-hand inverted repeats of Tn*21* (a mercury resistance transposon), respectively (38, 108). Within these borders were several ORFs, one with similarity to the MerR regulatory protein of the mercury reductase operon (134) and another with similarity to the *cmlA* (chloramphenicol resistance) gene product of Tn*1696* of plasmid R1033 from *P. aeruginosa* (11). However, the synthesis of polypeptides from these ORFs by an *E. coli*-derived protein synthesis system could not be detected. No equivalent of the mercury reductase gene, *merA*, was present in the DNA sequence; nor did the presence of the transposonlike element result in an increase in chloramphenicol resistance in *E. coli* (107). Also within the inverted repeat borders were two other ORFs which when translated had 88% amino acid identity to the 85% of N-terminal region of the Tn*21* resolvase (*tpnR*) and 78% identity to 15% of the C terminus of the Tn*21* transposase (130). The transposon-like element of pTF-FC2 is apparently defective in transposition and mercury resistance because of loss of genes, perhaps from an ancestral Tnp^+ Mer^+ transposon. There is still no phenotype for the *T. ferrooxidans* plasmid pTF-FC2.

Comparison of Plasmids

It is curious that the only two *T. ferrooxidans* plasmids that have been studied in any detail were isolated from strains originating from different parts of the world (Japan and South Africa), but both had features which resembled those of the IncQ plasmids. In the case of pTF1, the similarity was to the mobilization proteins that function in conjugation whereas in pTF-FC2 the similarity was to the proteins involved in plasmid DNA replication (Fig. 4). Plasmid pTF-FC2 appears to be a natural hybrid having replication functions like those of the IncQ plasmids and a mobilization region like those of the IncP plasmids.

Although *T. ferrooxidans* plasmids remain cryptic, studies with both pTF1 and pTF-FC2 have indicated that several plasmid *mob* and *rep* genes are expressed in *E. coli* and several other gram-negative bacteria. Plasmids isolated from *T. ferrooxidans* have been used in the construction of cloning vectors (46, 112, 114) and at least three plasmids based on *T. ferrooxidans* plasmids have been used to transform a *T. ferrooxidans* isolate (75, 131).

CLONING AND EXPRESSION OF GENES FROM *T. FERROOXIDANS*

An important approach to studying the genetics of *T. ferrooxidans* is to clone and examine the expression of *T. ferrooxidans* genes in other bacteria. The advantage of cloning genes from a genetically uncharacterized bacterium into well-characterized species such as *E. coli* or *Salmonella* species is the availability of a large variety of defined mutants. This permits the analysis of gene function and regulation by complementation. A disadvantage to this approach is that detailed studies of cloned genes in a heterologous background may not accurately represent the situation in the original bacterium. Furthermore, genes which are not present in bacteria such as *E. coli* but which are important in the metabolism of *T. ferrooxidans* cannot be studied in this way. This would apply to genes such as those involved in iron or sulfur oxidation. Studies of *T. ferrooxidans* genes in defined mutants of heterotrophic bacteria have nevertheless contributed greatly to our knowledge of the molecular biology of *T. ferrooxidans*.

Genes Involved in Nitrogen Metabolism

Although *T. ferrooxidans* is diazotrophic and very efficient at scavenging nitrogen from the atmosphere, the availability of nitrogen and the energy required for its fixation may limit bacterial growth and adversely affect the efficiency of leaching operations. The study of nitrogen metabolism in *T. ferrooxidans* is therefore of both fundamental and applied interest.

Glutamine synthetase. Under conditions of nitrogen limitation in bacteria which are able to fix nitrogen, glutamine synthetase (GS) plays a central role as it catalyzes one of the main reactions by which ammonia is assimilated (144).

$$\text{L-Glutamate} + NH_4^+ + ATP \rightarrow \text{L-glutamine} + ADP + P_i$$

The GS enzyme and the regulation of its structural gene (*glnA*) have been most extensively studied in *E. coli* and other enteric bacteria. In these bacteria the *glnA* gene is part of a

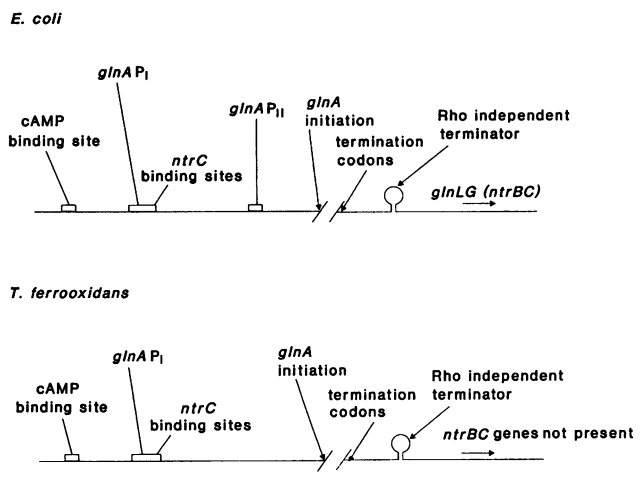

FIG. 5. Comparison of the arrangement of control elements and genes within the *glnA* operon of *E. coli* and *T. ferrooxidans*. The *glnA*pII (CTGGCACN$_5$TTGCA) type promoter and *glnLG* (*ntrBC*) genes are missing from the *T. ferrooxidans* operon. NtrC-binding sites overlap the *glnA*pI promoter.

complex operon (*glnALG*) whose transcription is regulated by the products (NR_I, NR_{II}) of the *ntrC* (*glnG*) and *ntrB* (*glnL*) genes, respectively (91). In *E. coli* synthesis of the enzyme is under the control of two promoters (Fig. 5) (118). Promoter *glnA* P_I is of the σ^{70} type, is responsible for low-level production of GS, and is subject to catabolite repression by the cyclic AMP-catabolite activator protein (CAP) complex. Expression from promoter *glnA* P_{II} is dependent on σ^{54} which is the product of the *ntrA* (*rpoN*, *glnF*) gene and is required for the transcription of promoters that have a characteristic −24 and −12 consensus recognition element (CTGGCAC-N$_5$-TTGCA). Transcription from *glnA* P_{II} is regulated by nitrogen and requires the phosphorylated *ntrC* gene product for full expression (61).

To determine whether the *glnA* gene from an acidophilic autotroph was similar to those of gram-negative heterotrophs, the *T. ferrooxidans glnA* gene was cloned, sequenced and its expression in *E. coli* studied (5, 110). The upstream region of the *T. ferrooxidans* gene contained several putative σ^{70}-type promoters but no σ^{54}-type promoter was identified. The upstream region also had a sequence resembling the CAP-binding consensus sequence and at least two NtrC-binding sites that overlap with the *glnA* P_I promoter. Like *E. coli*, the *T. ferrooxidans glnA* gene was followed by a Rho-independent terminator. The *T. ferrooxidans* GS was able to complement *E. coli* GS deletion mutants and the production of the *T. ferrooxidans* GS was regulated by nitrogen levels in *E. coli* (6). The *T. ferrooxidans* GS was also regulated at the protein level by an adenylylation mechanism similar to that of the family *Enterobacteriaceae*.

In contrast to the enteric bacteria, the *T. ferrooxidans glnA* gene was not linked to the equivalent of the *ntrBC* genes. The nucleotide sequence of the gene immediately downstream has been determined (66), and the *T. ferrooxidans ntrBC* genes have been cloned (see below). Both studies have confirmed that in the case of *T. ferrooxidans* the *glnA* genes and the *ntrBC* genes are unlinked.

Nitrogenase. Nitrogen-fixing bacteria possess a nitrogenase enzyme which catalyzes the reduction of atmospheric nitrogen to ammonia. Mackintosh (85) was the first to report that *T. ferrooxidans* was able to fix nitrogen by demonstrating the incorporation of $^{15}N_2$ label into cellular material. Using the *K. pneumoniae* nitrogenase structural genes (*nifHDK*) as a probe, Pretorius et al. (98) reported the presence of *nif* genes in five different *T. ferrooxidans* strains. The *nifHDK* genes from *T. ferrooxidans* ATCC 33020 were cloned and sequenced (97, 98, 105). There are two clearly identifiable sequences upstream of the *nifH* gene which are essential for the control of gene expression (97). These are a σ^{54}-dependent promoter and a binding site for the NifA activator approximately 100 bp upstream from the translation start. In addition, integration host factor has been shown to bind to the DNA sequence between the σ^{54}-dependent promoter and the NifA-binding site, thereby inducing DNA bending (49). This bending is thought to bring the NifA enhancer into contact with the RNA polymerase at the σ^{54} promoter. Examination of the *T. ferrooxidans* promoter region revealed a σ^{54}-dependent promoter and a pair of tandem consensus NifA-binding sites upstream from the start of the *nifH* gene. Hoover et al. (49) demonstrated that *E. coli* integration host factor was able to bind to the *T. ferrooxidans nifH* promoter region. The *T. ferrooxidans nifH* promoter was therefore very similar to the *nifH* promoters commonly found in typical heterotrophic bacteria.

The *nifHDK* genes and the *nifH* gene in particular have been sequenced from a large number of nitrogen-fixing bacteria. An alignment and comparison of the amino acid sequences of the *nifHDK* gene products with those from other bacteria has indicated that the nitrogenase of *T. ferrooxidans* is most closely related to isolates of the genus *Bradyrhizobium* (106, 158). This is interesting as 16S rRNA sequencing data clearly places *T. ferrooxidans* into the β-group of proteobacteria whereas the bradyrhizobia are members of the α-group.

An interesting feature of *T. ferrooxidans* physiology is that when the bacterium obtains its energy by the oxidation of ferrous iron, oxygen is the final electron acceptor and the organism is an obligate aerobe. Nitrogenase enzymes are, however, oxygen labile, and cells have elaborate systems to protect their nitrogenase from oxygen (96). When Mackintosh (85) demonstrated nitrogen fixation by *T. ferrooxidans*, she used ferrous iron as an energy source and found that the supply of oxygen was critical. Oxygen had to be provided in limited quantities, sufficient to allow enough iron oxidation to provide the energy for N_2 fixation but insufficient to inhibit nitrogenase activity. An understanding of how *T. ferrooxidans* is able to resolve the requirement of oxygen for energy generation, but its absence for N_2 fixation has still to be elucidated. Ferric iron has been shown to act as an electron acceptor during the oxidation of reduced-sulfur compounds and in the absence of oxygen (137). It is possible that N_2 fixation occurs during sulfur-ferric iron respiration, but this has not yet been tested.

***ntrA* sigma factor.** Attempts to clone the *T. ferrooxidans ntrA* gene by the conventional method which involves the complementation of *E. coli ntrA* mutants for the ability to grow on minimal medium plus arginine (141) failed. Berger et al. (9) devised a cloning strategy based on the σ^{54}-dependent expression of the formate-hydrogen-lyase pathway (10). This fermentative pathway results in the evolution of H_2 and CO_2 from formate and *E. coli ntrA* mutants are unable to produce gas. A cosmid gene bank was transformed into an *E. coli ntrA* mutant, and the transformants were examined for the ability to produce gas by using an agar overlay technique (9). This procedure resulted in the cloning of the *T. ferrooxidans ntrA* gene.

Sequencing data showed that the *T. ferrooxidans ntrA* gene had 49 and 50% amino acid identity to the σ^{54} of *K. pneumoniae* and *R. meliloti*, respectively (9). An ORF (ORF1) for a protein 241 amino acids was situated 12 bp upstream of the *T. ferrooxidans ntrA* gene. This protein showed 55% amino acid

sequence identity to an equivalent protein located immediately upstream of the *ntrA* gene of *R. meliloti* (1). The linkage of ORF1 to *ntrA* has also been reported for *K. pneumoniae* and *S. typhimurium* and therefore appears to be a structural feature of the *ntrA* locus of bacteria of very different physiological types. Although ORF1 has a clearly discernible ATP-binding site, the biological function of ORF1 and the reason for its linkage to *ntrA* are unknown.

A *lacZ* reporter gene fused to several σ^{54}-dependent promoters was used to demonstrate that the cloned *T. ferrooxidans ntrA* gene product was able to promote σ^{54}-dependent transcription in *E. coli ntrA* mutants. Complementation was however partial and insufficient to permit growth of an *E. coli ntrA* mutant on minimal medium plus arginine.

***ntrBC* genes.** The *ntrBC* genes from several bacteria such as *Azotobacter vinelandii* have been cloned by the simple technique of transforming a gene bank into an *E. coli ntrC* mutant followed by selection for growth on minimal medium plus arginine (141). An attempt to use this technique to clone the *T. ferrooxidans ntrBC* genes was not successful. A *T. ferrooxidans nifH* gene fused to a *lacZ* reporter gene and which was shown to be activated in the presence of a *ntrC* gene was used instead (66). Several plasmid clones were isolated which increased expression of the *nifH-lacZ* reporter fusion. Nucleotide sequencing confirmed the presence of the *ntrBC* genes. Genes that are normally subject to regulation by NtrB and NtrC were also regulated by the cloned *T. ferrooxidans ntrBC* gene products in *E. coli*.

Genes Involved in Fixation of Carbon Dioxide

T. ferrooxidans obtains its cellular carbon by the fixation of carbon dioxide via the Calvin cycle. The key enzyme in this cycle is D-ribulose-1,5-biphosphate carboxylase (RuBPCase). The RuBPCase from *T. ferrooxidans* has been reported to be of the form I type, a hexadecamer composed of eight large subunits (LSU) and eight small subunits (SSU) similar to those of higher plants and most photosynthetic bacteria (48). The apparent K_m values for the enzyme prepared from *T. ferrooxidans* Torma was similar to those from plants and algae but four- to fivefold lower than those of most other bacteria. Recently, Shively et al. (133) probed the chromosome of six *T. ferrooxidans* isolates with probes derived from *Anacystis nidulans rbpcL* gene for the form I enzyme and a part of the *Rhodospirillum rubrum rbpL* gene for the form II enzyme. Hybridization signals from restriction fragments of different sizes were obtained for both probes and *T. ferrooxidans* may therefore possess both form I and form II types of RuBPCase.

Kusano et al. (73) isolated the *T. ferrooxidans rbcL-rbcS* genes by using a hybridization probe from *Chromatium vinosum*. The cloned *T. ferrooxidans* genes were not expressed in *E. coli* unless placed under the control of a strong *tac* promoter. This resulted in the synthesis in *E. coli* of an enzyme with ribulose biphosphate-dependent CO_2-fixing activity which had a hexadecameric form like that of the native *T. ferrooxidans* RuBPCase. The newly cloned *T. ferrooxidans rbcL-rbcS* genes were used to identify and clone a second set (*rbcL2-rbcS2*) of RuBPCase genes from the same *T. ferrooxidans* strain. Both sets of genes were sequenced (75) and found to be absolutely identical. The perfect nucleotide sequence conservation extended from nucleotide −164, through the coding and intergenic regions of both subunits and up to 90 bp past the termination codon, a total of 2.0 kb. The DNA sequences further up- or downstream were substantially different. Northern (RNA) blot and primer extension experiments with mRNA isolated from *T. ferrooxidans* indicated that the *rbcL1-rbcS1* and/or *rbcL2-rbcS2* genes were cotranscribed although no region with all the elements and spacing of a σ^{70}-type consensus promoter could be identified. Amino acid sequence alignment with previously sequenced LSU and SSU from other bacteria showed that the *T. ferrooxidans* RuBPCase was much more closely related to the RuBPCase of the photosynthetic bacterium *C. vinosum* (LSU, 85.5% identity; SSU, 59.8% identity) than to the enzyme from any other photosynthetic bacterium, nonphotosynthetic autotroph, alga, or plant. The *rbcL* and *rbcS* genes have been cloned and sequenced from a different strain of *T. ferrooxidans* isolated in Chile (101). In addition, part of the LSU of a third *T. ferrooxidans* isolate was cloned and sequenced by PCR (43). All the data indicated that the *T. ferrooxidans rbc* genes have a closer phylogenetic relationship to the photosynthetic bacterium *C. vinosum* than to the chemoautotrophic bacterium *Alcaligenes eutrophus*.

Recently, a regulatory gene, *rbcR*, was found upstream of the *rbcL1* gene (72). The gene encodes a protein of 309 amino acids and is translated divergently with respect to *rbcL1*. Gel mobility shift assays, DNA footprinting, and site-directed mutagenesis experiments showed that *T. ferrooxidans* RbcR, produced in *E. coli*, binds specifically to overlapping promoter elements of the *rbcR* and *rbcL1* genes. The RbcR protein is thought to be a member of the LysR family of positive transcriptional regulators (42).

recA Gene

The RecA protein of *E. coli* plays an essential role in the induction of DNA repair (the SOS response) following damage by UV or chemical agents (149). It is also required for homologous recombination, during which it is able to facilitate the ATP-dependent assimilation of single-stranded DNA into homologous double-stranded DNA as well as the pairing and exchange of duplex DNA. The RecA protein is the product of the *recA* gene, which is subject to negative control by the *lexA* gene product. Genes that are repressed by LexA have clearly identifiable promoter sequences (149).

The *recA* gene from *T. ferrooxidans* has been isolated and sequenced, and its ability to carry out RecA-associated activities in *E. coli* has been studied (103, 104). *T. ferrooxidans* RecA was able to complement defects in DNA repair and genetic recombination in an *E. coli* strain from which the *recA* gene had been deleted. Although some RecA functions were restored to only 15% of the activity in the wild-type *E. coli* parent strain, the *T. ferrooxidans* RecA protein exhibited both DNA repair-associated and recombination activities. This included the induction of phage λ *fec* mutants and the induction of the *recA* gene of the *E. coli* HB101 *recA* mutant strain. In the HB101 mutant the *recA* gene is not subject to normal induction by DNA damage; however, in the presence of the *T. ferrooxidans recA* gene, induction of the defective *E. coli recA* gene following DNA damage was restored (103). The cloned *T. ferrooxidans recA* gene was expressed in *E. coli* only from a vector promoter. No sequences characteristic of the LexA-binding site could be identified upstream of the *T. ferrooxidans recA* coding sequence. This indicated that either a different type of promoter sequence must be present or, unlike *E. coli* or *P. aeruginosa*, the *T. ferrooxidans* gene is not the first gene of a transcriptional unit. The cloning of the *T. ferrooxidans recA* gene should facilitate the construction of mutants via marker exchange. No attempts to construct mutants of this type have yet been made. Recently, partial sequencing of the region downstream of the *T. ferrooxidans recA* gene has revealed a region with sequence homology to the gene for alanyl-tRNA synthetase, *alaS* (39). This linkage between the *T. ferrooxidans*

recA and *alaS* genes is similar to that in *E. coli*, *R. meliloti*, and *Rhizobium leguminosarum*.

leuB Gene

The *T. ferrooxidans leuB* gene, which encodes the 3-isopropylmalate dehydrogenase enzyme of the leucine biosynthesis pathway, was cloned by complementation of an *E. coli leuB* mutant (50). Expression of *leuB* in *E. coli* appeared to be from a *T. ferrooxidans* promoter and was repressed by leucine. The gene was sequenced and has approximately 50% amino acid sequence identity with bacteria as diverse as *S. typhimurium*, *Bacillus subtilis*, *Thermus aquaticus*, and *Lactococcus lactis*, as well as to the yeasts *Candida utilis* and *Saccharomyces cerevisiae* (60). The enzyme was purified and has characteristics similar to the equivalent enzyme from *S. typhimurium* and *Thermus aquaticus*, except that it has a broader substrate specificity and was able to utilize D-malate and L-malate in addition to 3-isopropylmalate (60).

Genes Associated with Resistance to Mercury

Mercury is highly toxic to most strains of *T. ferrooxidans*, and its presence in ore deposits may inhibit commercial leaching operations. Cinnabar (HgS) is the major mercury-bearing mineral in nature, and the presence of cinnabar has been shown to inhibit pyrite oxidation by mercury-sensitive *T. ferrooxidans* strains but not by a mercury-resistant strain (4). Mercury resistance is therefore important in some bioleaching processes.

The molecular biology of the bacterial mercury resistance system has recently been reviewed (134). In general the resistance mechanism is based on the reduction of mercuric (Hg^{2+}) ions to volatile elemental mercury (Hg^0). This is carried out by a mercury reductase enzyme, which is the product of the *merA* gene. Also involved are two or three other genes (*merT*, *merP*, and *merC*), whose products are involved in mercury transport, and a regulatory gene (*merR*), whose product binds mercury and acts both as a repressor (in the absence of Hg^{2+}) or an inducer (in the presence of Hg^{2+}).

The presence of mercury reductase activity in cell extracts of a mercury-volatilizing strain of *T. ferrooxidans* was first demonstrated by Olsen et al. (94). In a follow-up study, the enzyme from *T. ferrooxidans* was purified and shown to be similar in structure and function to the mercuric reductase isolated from other bacterial sources (14). Shiratori et al. (132) reported that 5 of 10 *T. ferrooxidans* strains isolated in Japan were resistant to more than 0.5 μg of mercuric chloride per ml. Chromosomal DNA from three of these resistant strains hybridized to a probe prepared from the Tn*501 mer* genes. This probe was used to isolate a plasmid clone that encoded proteins of 56 and 16 kDa. The 56-kDa protein was found to have 78 and 77% sequence identity with mercury reductase of Tn*501* and R100 (55), whereas the 16-kDa protein was shown to function as a mercury transport protein, MerC, and had 55% sequence identity to the *merC* gene product of R100 (71). Although the cloned *T. ferrooxidans merC* and *merA* genes were synthesized constitutively in *E. coli*, primer extension analysis with mRNA isolated from *T. ferrooxidans* indicated that the two genes were cotranscribed as an inducible operon in their natural host (53). A *merR* regulatory gene was not physically linked to the *merC* and *merA* genes as is typical for other mercury resistance systems. Instead, two distinct *merR* genes were found more than 6 kb distant from the *merC-merA* operon (54). The two *merR* genes were more than 3 kb apart and were separated by five potential ORFs. Two of these ORFs were identified as functional *merC* genes, two others appeared to be truncated

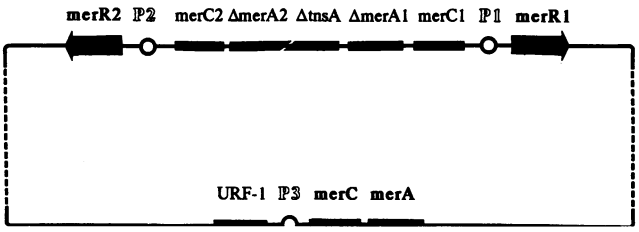

FIG. 6. Arrangement of the *mer* genes on the chromosome of *T. ferrooxidans* E-15. Functional *merC* (transport) and *merA* (mercury reductase) genes are transcribed from P3 and are not linked to a *merR* (regulator) gene. Two regulator genes, *merR1* and *merR2*, are located in separate operons and are transcribed in *T. ferrooxidans* from promoters P1 and P2. A gene duplication appears to have occurred such that a truncated, nonfunctional Tn*7* *tnsA* (transposase) gene is sandwiched between two truncated, inactive *merA* genes which are flanked by *merC1* and *merC2* genes which are functional in *E. coli*.

merA genes, and the fifth had 59% identity to 98 amino acids of the N terminus of the transposon Tn*7* transposase. The genes were arranged with a twofold axis of symmetry such that the truncated Tn*7* transposase was in the center bordered on either side by a truncated *merA* gene, a *merC* gene, and a *merR* gene (Fig. 6). Both *merR* genes were transcriptionally active in *T. ferrooxidans*, and the two *merC* genes both appeared to be functional in *E. coli*.

The genes associated with mercury resistance of *T. ferrooxidans* E-15 therefore differ from those of other bacteria in several important respects. The genes are located on the chromosome rather than on a plasmid, and the operon structure is very different. A clue to the duplication of the dislocated *merR* and *merC* genes may be provided by the truncated ORF that has homology to a Tn*7*-like transposase (54). The mercury resistance genes may have become inserted into the chromosome as the result of a multiple transposition event. Evidence in support of this is that the codon usage pattern for the functional *merA* gene is similar to that of the mercury resistance transposon Tn*501* and plasmid R100 but substantially different from other *T. ferrooxidans* chromosomal genes (55, 117). The arrangement of the mercury resistance genes in other *T. ferrooxidans* strains is unknown.

It is interesting that another *merR* gene was identified on the broad-host-range plasmid pTF-FC2 isolated in South Africa. This plasmid does not have any other *mer* genes, and it is possible that this *merR* gene either is not functional or is a regulatory gene of a yet to be identified chromosomal resistance determinant. Silver and Walderhaug (134) produced a phylogenetic tree of sequence relationships between MerR proteins from different sources. As may be expected, the two *T. ferrooxidans* chromosomal MerRs were the most different MerR sequences from gram-negative sources, whereas the predicted MerR-like sequence from the pTF-FC2 plasmid was still more different from any of the MerRs from either gram-negative or gram-positive sources.

Genes Involved in Energy Production

A major challenge to molecular biologists who work with *T. ferrooxidans* and related chemoautolithotrophic bacteria is to study the structure, expression, and regulation of the genes associated with the physiological characteristics that have made these bacteria industrially important. These are the genes that synthesize the proteins required for the oxidation of ores and, particularly, those involved in the oxidation of iron and sulfur compounds. Because iron and sulfur compounds are

oxidized primarily to gain energy, a study of the molecular biology of the process of energy production is relevant.

Relatively few bacteria can oxidize sulfur, and even fewer can oxidize iron. There are no defined mutants with mutations in iron or sulfur oxidation pathways, and only rudimentary genetic systems for some strains have been developed. This means that screening gene banks by complementation of mutants is not yet possible. Furthermore, until recently, no DNA probes for iron and sulfur oxidation genes existed. Identification of the proteins involved in iron and sulfur oxidation is itself a challenge. It has, however, been shown that if total protein is extracted from a *T. ferrooxidans* strain grown on either an iron or sulfur medium and the two protein profiles are compared, clear differences are seen (90, 95). Some proteins are produced when the bacterium is grown on iron but not on sulfur, and others are synthesized only when the bacterium is grown on sulfur. Once these iron- or sulfur-induced proteins have been identified, they can be purified and antibodies can be raised. Alternatively, a short stretch of the amino acid sequence can be determined and a DNA oligonucleotide hybridization probe can be synthesized. These antibodies or oligonucleotides may be used to screen *T. ferrooxidans* gene banks.

T. ferrooxidans can grow on a variety of reduced-sulfur compounds, and the enzymology of this process has been studied in an effort to work out the oxidation pathway (62, 68). There is still much to be done before the pathway is resolved, and little is known of the molecular genetics of the sulfur oxidation pathway in this bacterium or other thiobacilli. Because no genes of the sulfur oxidation pathways have been identified or cloned, this will not be discussed further. Considerably more is known about the mechanism of iron oxidation.

Iron oxidation. Several of the components involved in the oxidation of iron by *T. ferrooxidans* have been identified (12, 51). These include a 92-kDa outer membrane porin (90), a small blue copper protein called rusticyanin, an uncertain number of cytochromes of the *c* and *a* types, and an Fe(II) oxidase. A diagram of the likely spacial relationship between these components is shown in Fig. 7. The redox potential of the Fe^{2+}/Fe^{3+} redox couple is 0.77 V, whereas that of the electron-accepting O_2/H_2O couple is 0.82 V at pH 7 and 1.12 V at pH 3.2. Since the internal pH of *T. ferrooxidans* cells is approximately 6.5, the amount of energy available if the terminal reduction of O_2 took place in the more acidic environment outside the cytoplasmic membrane is considerably greater than if this reaction took place in the cytoplasm. This possibility of oxygen being reduced outside the cell was first suggested by Cox and Brand (24) and is supported by the studies of Yamanaka and coworkers, who purified cytochrome c_{552} and found it to be reduced by a membrane fraction of *T. ferrooxidans* more rapidly at pH 3.0 than pH 6.5 (125). Subsequently they isolated the cytochrome oxidase of *T. ferrooxidans* and reported it to have a pH optimum of 3.5 (59). In contrast, Ingledew (52) has argued that the heme of the cytochrome a_1-type oxidase of *T. ferrooxidans* is deeply embedded in the cytoplasmic membrane and that the generation of a transmembrane proton electrochemical potential is determined by the difference in redox potential between the electron donor and acceptor in the bulk phase (51).

(i) Fe(II) oxidase. An Fe(II)-oxidizing enzyme of 63 kDa was purified to electrophoretic homogeneity (37). This Iro enzyme rapidly reduced *T. ferrooxidans* ferricytochrome c_{552} in the presence of Fe^{2+} ions and under acidic conditions. After boiling, the enzyme dissociated into 8 to 10 6-kDa subunits. A degenerate oligonucleotide probe was synthesized from a partial amino acid sequence of the subunits and was used to clone the *iro* gene (77). The gene was sequenced, and the Iro protein was found to consist of 90 amino acids including a possible 37-residue membrane transport signal sequence. Comparison of the derived sequence with current data bases indicated a clear relationship with the high-redox-potential iron-sulfur proteins (HiPIP) of *Rhodopseudomonas marina* (48% amino acid identity), *Rhodopseudomonas globiformis* (51%), and *Rhodomicrobium vannielii* (47%). The HiPIPs are a group of soluble bacterial ferredoxins which are commonly found in the purple photosynthetic bacteria and contain a single redox-active four iron-four sulfur cluster in a peptide chain of 54 to 85 residues. The *T. ferrooxidans* Iro appears to be a new member of this family of proteins, which function in electron transport but generally do not show enzyme activity. Northern blot RNA-DNA hybridization and mRNA primer extension analysis with *T. ferrooxidans* mRNA indicated that the *iro* gene was transcribed independently of any other gene. Interestingly, a gene with high similarity to the *purA* gene of *E. coli* and which could functionally complement *E. coli purA* mutants was located 105 bp upstream of the *T. ferrooxidans iro* gene (Fig. 8) (76). In *E. coli* the *purA* gene is followed by two ORFs, ORF-I and ORF-II. In *T. ferrooxidans* an ORF with

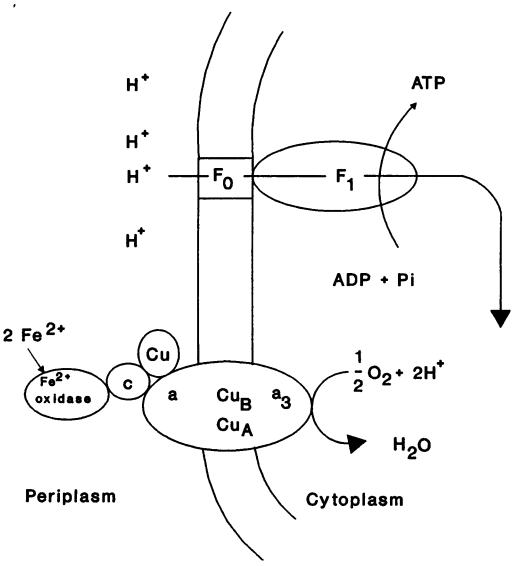

FIG. 7. Possible arrangement for the Fe^{2+} oxidation electron transport system of *T. ferrooxidans* in accordance with the proposal of Yamanaka et al. (154). Fe^{2+} oxidase, *c*, and Cu are Fe(II)-cytochrome c_{552}, soluble cytochrome c_{552}, and rusticyanin, respectively. *a*, a_3, Cu_A, and Cu_B are likely components of a terminal oxidase. F_0 and F_1 are the membrane-integral and membrane-associated portions, respectively, of ATP synthase.

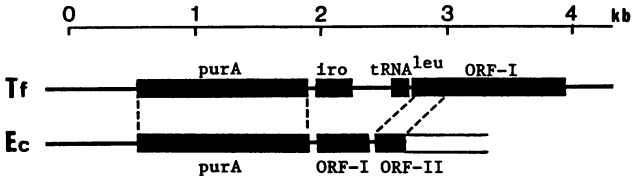

FIG. 8. Comparison of the organization of the *purA* gene and flanking regions from *T. ferrooxidans* (Tf) and *E. coli* (Ec). Coding regions are represented by thick solid bars, and regions of significant amino acid sequence similarity are represented by parallel broken lines. Reproduced from reference 76 with permission of the publisher.

homology (57%) to the *E. coli* ORF-II but not to ORF-I was present. The *iro* gene and a gene for a leucyl-tRNA were sandwiched between the *purA* gene and the *T. ferrooxidans* equivalent of ORF-II (76).

(ii) Rusticyanin. Rusticyanin is a 16.5-kDa soluble blue copper protein which is found in abundance in the periplasm of *T. ferrooxidans* growing on iron (21, 23). Levels of rusticyanin have been shown to be reduced when the bacterium is growing on medium containing elemental sulfur as the energy source (56). The protein has been purified and has an unusually high redox potential of +680 mV. Its exact role in the iron-dependent respiratory electron transport chain remains unclear (13). Ingledew and coworkers raised antibodies against rusticyanin which were used by Kulpa et al. (69) to screen a *T. ferrooxidans* genomic library. Similar studies were reported by Jedlicki et al. (56). Although both groups of researchers reported the tentative isolation of the rusticyanin gene, these studies have not been taken further. The amino acid sequence of rusticyanin has been determined from several strains of *T. ferrooxidans* by direct protein analysis (93, 121), and attempts are in progress to reclone the gene. There appears to be only a single copy of the rusticyanin gene per chromosome (102).

Proton-translocating ATP synthase genes. A result of the oxidation of ferrous ions or reduced-sulfur compounds by *T. ferrooxidans* is the generation of a transmembrane pH gradient (ΔpH) (20). The synthesis of ATP by using the potential of this transmembrane ΔpH has been directly demonstrated with *T. ferrooxidans* membrane vesicles (3). Investigation of the *T. ferrooxidans* H^+-translocating ATP-synthase is particularly interesting because although the organism grows optimally within the pH range 1.5 to 3.5 (20, 51), the cytoplasmic pH is close to neutral. Cox et al. (25) reported that when *T. ferrooxidans* is growing on Fe^{2+} at pH 2.0, the cells maintained a ΔpH of 4.5 units. A study of what modifications to the ATP-synthase have occurred to allow for use of this unusually large ΔpH could provide fundamental insights into the functioning of the enzyme and also the acidophilic nature of the bacterium.

The H^+-translocating ATP synthases of bacteria, mitochondria, and chloroplasts consist of two parts, the membrane-integral F_0 portion and the membrane-peripheral F_1 cytoplasmic portion (35). In *E. coli* the F_0 portion is composed of three subunits and contains the channel through which protons are translocated, while the F_1 portion is composed of five subunits and is the ATP-synthesizing or ATP-hydrolyzing catalytic portion (Fig. 9). The structure of the *E. coli atp* (also called *unc* or *pap*) operon and polypeptide subunits has been extensively reviewed (35, 129, 150).

Dozens of *E. coli* mutants defective in the genes for the different ATP synthase subunits have been isolated. These mutants are able to grow on minimal medium containing glucose but not on medium containing succinate as the sole energy source. Numerous clones capable of complementing the *E. coli* F_1 mutants, but none that could complement F_0 mutants, were isolated (18). A clone containing all five of the *T. ferrooxidans atp* F_1 genes and two of the *atp* F_0 genes (b and c) was identified and the *atp* genes were sequenced. The five subunits of the F_1 portion of the *T. ferrooxidans* ATP synthase were shown to form a functional association with the subunits of the *E. coli* F_0 portion in an *E. coli* strain deleted for the entire *atp* operon. A hybrid enzyme in which three of the five F_1 subunits were from *E. coli* and two from *T. ferrooxidans* was also found to be functional. However, a clone that produces the *T. ferrooxidans* F_0 c and b subunits from a vector promoter could not complement the corresponding *E. coli* mutants. The ability of the ATP synthase to function in the presence of a large ΔpH therefore appears to reside in the subunits of the F_0 portion. Several unusual substitutions in domains of the F_0 c subunit that have been implicated in proton translocation and could contribute to the gating properties of an acidophilic ATP synthase were found.

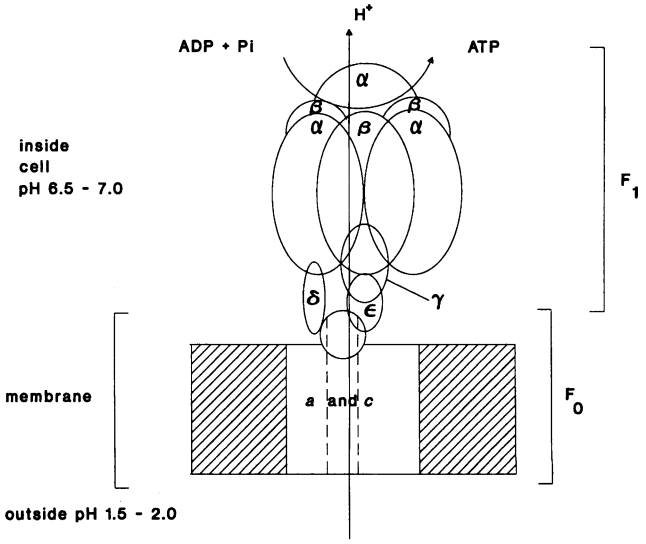

FIG. 9. Arrangement of the subunits of the *E. coli* ATP synthase, as proposed by Walker et al. (150). Because of its acidophilic nature, *T. ferrooxidans* has an unusually large pH gradient across the membrane.

Repeated Sequences, IS elements, and Transposition

Holmes and coworkers used Southern hybridization experiments with cloned pieces of *T. ferrooxidans* ATCC 19859 chromosomal DNA as a method of distinguishing between different strains (157), when they discovered two families of repeated sequences (156). Both families of repeated sequence were 1.3 to 1.4 kb in size and were repeated 20 to 30 times per chromosome. These authors estimated that approximately 6% of the chromosome of strain ATCC 19859 consisted of repetitive DNA. Since the two families of repeated sequences reported would account for less than half of this amount, it is likely that other families of *T. ferrooxidans* repeated sequences exist. The repeated sequences appear to be widespread. In a survey of 22 *T. ferrooxidans* isolates, both families of repeated sequences occurred in 12 strains, one of the families but not the other occurred in 4 strains, and the other 6 strains had neither family. Furthermore, two of three strains of *T. thiooxidans* examined also contained both repeated sequence families (45). Both families of repeated sequences have been examined in some detail, and both have structural features of insertion sequences (IS elements).

A representative of the family, IS*T2*, has been sequenced and found to be 1,408 bp long (155). It has two imperfectly conserved, 25-bp inverted repeats at either end of the IS element and two 9-bp target site duplications immediately adjacent to the inverted repeats (Fig. 10). The ends of a second IS*T2* element were sequenced and found to have identical inverted repeats and similarly sized target duplications to those of the first. Three ORFs, of which the longest was 888 bp, were located within IS*T2*. One of the ORFs has significant sequence similarity to the putative transposases of the IS*Rm3* insertion sequence from *Rhizobium meliloti* and IS*256* from *Staphylococcus aureus* (151).

A member of the second family of repetitive DNA, IS*T1*, has

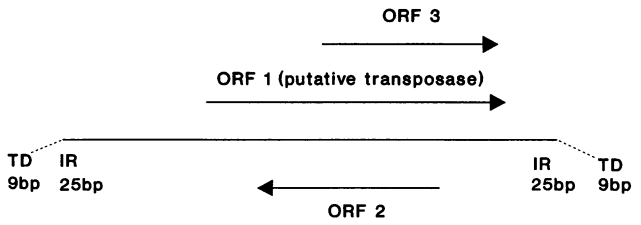

FIG. 10. Structure of the *T. ferrooxidans* insertion sequence IS*T2*. TD represents the target site direct repeats, and IR represents the insertion sequence inverted repeats.

also been partially characterized (159). IS*T1* is about 1.3 kbp in size, has 26-bp imperfectly paired inverted terminal repeats, and 5-bp target duplications. It also contains two ORFs which could encode proteins of 396 and 156 amino acids but has little homology with other known repeated sequences.

Both the IS*T1* and the IS*T2* insertion sequences appear to be mobile. When the Southern hybridization experiments were repeated after several generations of propagation in the laboratory, slightly different banding patterns with IS*T1* and IS*T2* probes were obtained (45, 47). This showed that both the families of repeats were mobile and presumably actively transposed within *T. ferrooxidans*.

Phenotypic Switching within *T. ferrooxidans*

When *T. ferrooxidans* is cultivated on a solid medium containing a mixture of iron and thiosulfate as an energy source, colony morphology variants are detected at high frequency (127). Large, spreading, highly motile colonies which lack the ability to oxidize iron but retain the capacity to oxidize thiosulfate or tetrathionate arise spontaneously. Chromosomal banding patterns obtained with the IS*T2* element as a probe clearly indicated that these large-colony variants originated from *T. ferrooxidans* and were not culture contaminants. The variants arose from and reverted to wild-type iron oxidizers (45) at frequencies that were several orders of magnitude greater than the frequency associated with point mutations. Schrader and Holmes (127) suggested that preliminary evidence showed that specific changes in the position of the mobile elements were associated with the formation of the large-colony variant. Furthermore, these specific changes returned to a wild-type pattern in the wild-type revertants (45). They tentatively proposed that the transposition of IS elements in *T. ferrooxidans* may be the causative mechanism which gives rise to the large-colony variant and its reversion. Whether the transposition is programmed in the same sense as phase variation in *Salmonella* species or antigen switching in *Neisseria gonorrhoeae* or whether transposition is random is unknown.

Several issues in regard to *T. ferrooxidans* phenotypic switching remain to be resolved. On reversion of the large-colony variant to wild type, the bands of some of the family 1 repeated sequences returned to the wild-type position but others remained in their large-colony-variant positions (45). The mobility of the IS*T2* elements during the formation of colony variants and what happens on reversion have not been investigated. In addition, *T. ferrooxidans* ATCC 33020 does not have either family of repeated sequence (45) but is nevertheless capable of forming large-colony variants (127). It is possible that strain ATCC 33020 has a different family of nonhomologous (and as yet undiscovered) mobile repeated sequences, but this has not been shown.

T. ferrooxidans is noted for its remarkable adaptability to changing conditions. It has been suggested that the adaptation of strains to adverse conditions (see above) and the presence of mobile repeated sequences may be associated. Although a linkage has not been proven, Schrader and Holmes (127) have speculated that the genetic instability associated with mobile genetic elements and the phenomenon of phenotypic switching may introduce an added flexibility in response to changing environmental conditions. This flexibility may well permit some members of the population to survive under conditions which would prevent growth of the majority (127).

Additional Genes

A number of other genes from *T. ferrooxidans* have been cloned and in some cases sequenced, but work on these has either ceased or the investigation is at an early stage.

rRNA genes. rRNA genes encoding the 16S and 23S rRNAs have been isolated (122). Hybridization experiments indicated that these genes are arranged in an operon similar to the rrnB operon of *E. coli* and that in *T. ferrooxidans* there are two sets of these genes. The two operons were named $rrnT_1$ and $rrnT_2$, and the promoter region of $rrnT_2$ was sequenced (138). Several features characteristic of the *E. coli rrn*-type promoters were found. These include a near-consensus σ^{70} promoter, a discriminator sequence like that involved in the *E. coli* stringent response and antiterminator elements. The spacer region between the 16S and 23S rRNA has also been sequenced and found to contain the genes for isoleucine- and alanine-tRNA (147). Short (17- to 20-bp) oligonucleotides have been synthesized from different regions of the *T. ferrooxidans rrn* operon and used to design probes to enable researchers to identify thiobacilli and specifically recognize certain *T. ferrooxidans* strains (123).

RNase P. The *rnpB* gene encoding the tRNA-cleaving enzyme RNase P was fortuitously isolated and sequenced (139). The potential structural sequence was 70% homologous to the corresponding genes from *E. coli* and *S. typhimurium*. In regard to computer-predicted secondary structure, the RNase P of *T. ferrooxidans* most closely resembles those of the proteobacterial γ-group such as *E. coli* and *C. vinosum* (17).

Sulfur assimilation. A *T. ferrooxidans* gene bank was screened for the ability to genetically complement *E. coli* mutants defective in specific steps of the sulfur assimilation pathway. By using this technique, a clone which complemented *E. coli cysD* and *cysC* mutants was identified (36). These two genes encode the enzymes for the first two steps of sulfate activation prior to reduction and assimilation to produce cysteine. Protein gels of the in vitro translation products from the cloned DNA indicated that *cysN* was located between *cysD* and *cysC*. The operon structure appeared to be similar to the first three genes of the *cysDNCHIJ* operon of *E. coli* (81). Since *T. ferrooxidans* is able to obtain energy via the oxidation of sulfur, the regulation of sulfur metabolism is particularly interesting. The bacterium is faced with the choice between the oxidation of sulfur for energy production (dissimilatory pathway) or the reduction of sulfur for biosynthesis (assimilatory pathway). It is not known exactly how the flow of sulfur to these two pathways is regulated.

GENE TRANSFER STUDIES

In the absence of confirmed reports of the occurrence of bacteriophages and numerous unsuccessful attempts at producing a state of transformation competence, conjugation and electroporation are the most likely means of transferring DNA into *T. ferrooxidans* cells.

Conjugation Systems

Although no self-transmissible *T. ferrooxidans* plasmids have been isolated, at least three *T. ferrooxidans* plasmids have been shown to be mobilized between *E. coli* strains (115). Two of these have been studied in some detail (see the sections on plasmid pTF1 and plasmid pTF-FC2, above) (Fig. 2 and 4), and the presence of *mob* genes has been confirmed. It is therefore highly likely that a natural conjugation system exists between *T. ferrooxidans* strains and possibly between *T. ferrooxidans* and other bacteria (since one of the plasmids is a broad-host-range plasmid). Attempts to transfer plasmids such as the broad-host-range IncP plasmids RP4 and R68.45 directly from *E. coli* to *T. ferrooxidans* have not been successful. However, plasmids of the IncP and IncW incompatibility groups could be mated from *E. coli* to facultatively autotrophic thiobacilli such as *T. novellus* (26) and *T. neapolitanus* (70). A two-step mating procedure has been attempted: in the first step, plasmids were mated from *E. coli* into facultative thiobacilli on heterotrophic media at near-neutral pH, and in the second step the thiobacilli were mated with *T. ferrooxidans* on inorganic medium at low pH (114). Although several plasmids have been mated into a number of facultative thiobacilli, no transfer of the plasmids into *T. ferrooxidans* has been detected.

A report that IncP plasmids can be conjugated directly from *E. coli* to *T. thiooxidans* (58) has recently been published. If this work can be repeated, it would be encouraging for the development of a *T. ferrooxidans* conjugation system. Like *T. ferrooxidans*, *T. thiooxidans* (64) is obligately autotrophic, highly acidophilic, and, from 16S RNA sequence data, closely related to *T. ferrooxidans* (78).

Electroporation

Electroporation is a transformation technique that has been increasingly applied to a wide range of bacteria for which no natural genetic transfer system has been found. Kusano et al. constructed shuttle vectors consisting of the *T. ferrooxidans mer* resistance determinant cloned into four natural *T. ferrooxidans* plasmids, as well as into pKT240, a plasmid containing an IncQ-type replicon. These plasmids were electroporated into mercury-sensitive *T. ferrooxidans* cells, the *mer* operon was induced, and the cells were plated onto medium containing inhibitory concentrations of mercury (74). Of 30 strains electroporated, only 1 gave transformants at a low efficiency of 120 to 200 colonies per μg of plasmid DNA. (Usual electroporation frequencies are approximately 10^6/μg DNA.) Transformants were obtained with the pKT240 construct and with three of the four *T. ferrooxidans* plasmid-based vectors tested. The efficiency of electroporation was independent of the shuttle vector used. Recombinant plasmids were reisolated from the transformed cells, and the restriction enzyme cleavage patterns indicated that no plasmid rearrangements had occurred.

To prove that the transformed plasmid DNA had been propagated in *T. ferrooxidans*, methylated DNA to be used for electroporation was prepared from an *E. coli dam*$^+$ *dcm*$^+$ strain. The plasmid DNA isolated from the *T. ferrooxidans* transformants was tested to determine whether it had been modified during replication by digestion with methylation-sensitive enzymes *Mbo*I and *Eco*RII. Whereas the DNA prepared from the *E. coli dam*$^+$ *dcm*$^+$ strain was resistant to these enzymes, the recombinant DNA from *T. ferrooxidans* was readily digested. The interpretation of this observation was that replication of the plasmids in *T. ferrooxidans* had occurred and that during replication the DNA had not been modified. The recombinant plasmids were stable in *T. ferrooxidans* for as many as 110 generations without selective pressure (74).

CONCLUSIONS AND FUTURE RESEARCH

As a result of its acidophilic, chemoautolithotrophic mode of life and its inhibition by organic compounds, *T. ferrooxidans* occupies an ecological niche populated by relatively few other bacterial species. In such a situation it may be expected that the bacterium would have had little opportunity for genetic exchange with other soil microorganisms. The possibility therefore exists that *T. ferrooxidans* was subjected to a large amount of genetic drift compared with the more extensively studied heterotrophic bacteria. An unusually large amount of genetic drift does not appear to have occurred. Although 16S rRNA data place the majority of *T. ferrooxidans* and *T. thiooxidans* strains in a distinct group on their own, these bacteria clearly belong to the division *Proteobacteria*, and the group is best placed close to the junction of the β and γ subdivisions (78). The finding of a broad-host-range mobilizable plasmid in *T. ferrooxidans* may indicate that the organism is not as genetically isolated as might have been expected.

A large number of *T. ferrooxidans* genes have been isolated by functional complementation of *E. coli* mutants. In some cases the *T. ferrooxidans* genes or enzymes have been regulated by or been able to regulate their counterparts in *E. coli*. For example, the regulation by adenylylation of the *T. ferrooxidans* glutamine synthetase in *E. coli* (6). Another is the ability of the *T. ferrooxidans* RecA protein to induce the *E. coli* SOS response or to inactivate the phage λ repressor (103, 104). In other cases, complementation has been only partial. For example, the *T. ferrooxidans ntrA* gene product was able to complement expression of the *E. coli fdh* genes but did not permit *E. coli ntrA* mutants to grow on minimal medium plus arginine (9). The functionality of many *T. ferrooxidans* gene products in *E. coli* is convincing evidence that the internal pH and cytoplasmic milieu of the two bacteria are similar. There is therefore a good probability that genes and enzymes from other bacteria will function in *T. ferrooxidans*. Some of these, such as genes for metal ion resistance, may have practical significance.

A major problem is the lack of an easily usable genetic system for the bacterium. Although the successful electroporation of a *T. ferrooxidans* strain is a major breakthrough, a number of difficulties persist. For example, only 1 of 30 *T. ferrooxidans* strains tested was transformed, and this occurred at a very low frequency (74). Selection for mercury resistance was not a very stringent selection procedure, and only 13 of 22 colonies which grew on mercury contained the recombinant plasmid. A good selectable marker for use with *T. ferrooxidans* is still lacking. Part of the problem is that *T. ferrooxidans* cells take a long time (about 10 to 14 days) to form colonies on solid medium and many strains will not form colonies at all. The low pH of the medium, extended incubation times, inherent resistance, and the occurrence of spontaneous mutations which confer resistance to substances such as tetracycline or arsenic (7) mean that many of the commonly used markers are not suitable for use with *T. ferrooxidans*. There are as yet no defined chromosomal mutants which would allow cloned *T. ferrooxidans* genes to be used for selection. The construction of mutants of this type would be of great value. Natural plasmids are possible sources of genetic markers which would be expected to function *T. ferrooxidans*. Identification of a marker from this source could provide a possible solution to the lack of selectable genetic markers.

An important question for *T. ferrooxidans* researchers to consider concerns the aspects of research into the molecular biology of the bacterium that are likely to be of practical value. For example, the high-rate arsenopyrite biooxidation processes

have several important requirements that must be fulfilled for biooxidation to proceed efficiently. These are the need for arsenic resistance, the need for cooling, and the need for a continuous upward adjustment of the pH as a result of microbial acid production and the addition of small amounts of inorganic nutrients (146). Savings could be achieved if the bioleaching bacteria could grow faster, at higher cell densities, at higher temperatures, at lower pH, and with even smaller quantities of added nutrients. Attempts to operate a high-rate arsenopyrite biooxidation process at temperatures higher than 45°C have not been successful. Raising the operating temperature above 45°C may be possible only if thermophilic bacteria and not *T. ferrooxidans*-dominated populations are used (92). A study of the biology of pH tolerance such as the maintenance of the transmembrane pH gradient could be of practical significance. Molecular research on the factors affecting *T. ferrooxidans* nutrition is another topic with possible practical consequences. Investigations into carbon dioxide fixation, nitrogen assimilation, phosphate metabolism (57, 128), and the assimilation of sulfur (36) have already been initiated. Some of this research is still in the most elementary stages and must be extended. Potentially rewarding research is likely to come from an understanding of the molecular biology of iron and sulfur oxidation. The value of this work to the industrial process is that iron is oxidized in preference to sulfur in mixed iron pyrite or arsenopyrite ores. In gold-bearing arsenopyrite biooxidation, the sulfur accumulates in the system and coats the particles. This reduces the rate of oxidation. As discussed above, this work is not easy and a long-term cooperative research effort among several laboratories currently working on individual proteins is required to make good progress.

It has been approximately 10 years since the first results of investigations into the molecular biology of *T. ferrooxidans* were published. The bacterium is slow growing and difficult to work with, and the research has been carried out in relatively few laboratories. Nevertheless, substantial progress in our understanding of the molecular biology of the bacterium has been made. If the progress of research in the second decade is as productive as that in the first, the use of *T. ferrooxidans* and other related chemoautolithotrophs is likely to make an even greater impact and change the face of the mining industry for good.

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