CONF-890819S--1

UCRL-102413
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This paper was prepared for submittal to:
Proc. of: *Biohydro-metallurgy '89 Symposium,*
Jackson Hole, WY, Aug. 13-18, 1989

27 November 1989

Lawrence Livermore National Laboratory

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UCRL--102413

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Cloning and Characterization of *Thiobacillus ferrooxidans* Genes Involved in Sulfur Assimilation

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ABSTRACT

The sulfate activating locus from Thiobacillus ferrooxidans has been cloned and expressed in Escherichia coli. The genes encoding ATP sulfurylase (ATP: sulfate adenylyl transferase, EC 2.7.7.4) and APS kinase (ATP: adenosine 5'-phosphosulfate 3'-phospho transferase, EC 2.7.1.25) have been isolated from a T. ferrooxidans genomic library constructed in pBR322. The approach used involved the utilization of mutants of E.coli defective in specific steps of the sulfur assimilatory pathway to isolate and identify, by genetic complementation, the corresponding genes and gene products in T. ferrooxidans.

In E. coli, the genes of sulfur assimilation are found in a locus mapping at 59 minutes of the chromosome. Included here are a cluster of genes organized in an operon designated cysDCHIJ. The proteins encoded by these genes include: ATP sulfurylase, APS kinase, PAPS reductase and sulfite reductase respectively. A plasmid designated pTFcys13 which contains a 4.7 kb T. ferrooxidans insert was found to complement both cysD$^-$ and cysC$^-$ E. coli mutants. pTFcys13 did not complement E. coli mutants carrying either cys I, cysJ or cysH defective genes. The genetic organization of the sulfate reducing locus has been inferred from deletion analysis of the insert in pTFcys13 coupled to complementation studies. Most deletions obtained led to a complete loss of ATP sulfurylase activity pointing to the possibility that more than one gene may encode this enzyme and thus, to its probable multimeric nature. The multimeric make-up of the sulfurylase was verified by in vitro transcription-translation studies with the cysD-complementing plasmid that indicated the enzyme is made up of two non-identical subunits with an estimated size of 36 and 62 kD.

INTRODUCTION.

The sulfur metabolisms of T. ferrooxidans constitutes an interesting area of study. Not only can this organism oxidize a variety of sulfur compounds in energy yielding reactions, but it is also capable of reducing sulfate in assimilatory fashion through a pathway organized to incorporate sulfur atoms into the sulfur-containing amino acids cysteine and methionine. Thus, the T. ferrooxidans cell must posses the potential and the capability to regulate the synthesis of enzymes involved in diametrically opposed chemical reactions. Sulfate production in one case, and sulfate reduction in the other (figure 1 A and B). Although a great deal is known about the nature of the biochemical steps involved in the oxidation of sulfur compounds in T. ferrooxidans (Silver and Lundgren, 1968 a and b), scant information is available on the sulfate assimilatory pathway.

Sulfate is taken up and assimilated by a variety of organisms to form the sulfur-containing amino acids cysteine and methionine (Schiff and Hodson, 1973). The first enzyme in the sulfate assimilation pathway, ATP sulfurylase, catalyses the activation of intracellular sulfate by ATP.

$$\text{ATP} + \text{Sulfate} \longrightarrow \text{APS} + \text{PP}_i$$ [1]

In E.coli the gene encoding this enzyme (cysD) is subject to regulation. When cysteine is provided in the growth medium,the synthesis of the enzyme is repressed whereas supplying the cells with a poor sulfur source, such as reduced glutathione or djenkolic acid,leads to its derepression (Jones-Mortimer, 1968; Kredich, 1971)..

The second enzyme in this pathway, APS kinase, catalyses the phosphorylation of APS.

$$\text{APS} + \text{ATP} \longrightarrow \text{PAPS} + \text{ADP}$$

The gene (cysC), encoding the enzyme that catalyzes this step, is coordinately regulated with cysD. Both in E. coli and Salmonella typhimurium, these genes reside adjacent to each other in the chromosome and together with cysH, cysI, cysJ, make up one of several cys custers present in these organisms (Jones-Mortimer, 1973; Leyh et al., 1988).

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[1] Abbreviations used: APS, adenosine 5'-phosphosulfate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; PP_i, inorganic pyrophosphate; CMM, Clostridium minimal medium

In this paper we present work which describe the isolation of genes involved in the sulfate assimilation of T. ferrooxidans, their genetic organization and the identification of their protein products.

METHODS

MATERIALS AND METHODS

Bacterial Strains and plasmids are described in Table 1

Culturing Methods. T. ferrooxidans was grown using the 9K iron medium of Silverman and Lundgren (1959). The assay and media conditions used for the determination of ATP sulfurylase and ATP kinase enzymatic activities have been described (Hanna and Taylor 1988; Fry et al. 1989).

Gene library Construction and selection of clones expressing ATP sulfurylase activity.

The preparation of a T. ferrooxidans genomic library has been described previously (Fry et al., 1988). Selection of plasmids from the library expressing ATP sulfurylase and APS kinase activity was carried out by genetic complementation of strains JM221 and JM81A, a cysD$^-$ and cysC$^-$ E. coli mutant respectively. The complementation studies were performed by transforming the appropriate strain with DNA isolated from pools of the entire 3,000-member genomic library and selecting for simultaneous ampicillin resistance and cysteine independence.

DNA manipulations. Ligations, plasmid deletions and other DNA manipulations were carried out using standard techniques of recombinant DNA (Maniatis, 1985; and Perbal, 1988)

Nick translation. The probe was labeled with ^{32}P-dCTP using a nick translation kit purchased from New England Nuclear. Prehybridization and hybridizations were carried out overnight at 65 C° essentially as described by Southern (1975).

In vitro transcription-translations. Cell-free transcription translation of plasmids pTFcys13, pBScys13ΔHindIII and its respective derivatives was done using a Prokaryotic In Vitro Transcription-Translation kit purchased from Amersham.

RESULTS

Complementation of mutants and identification of genes

The approach used in these studies relied on genetic complementation of available mutants of E. coli, defective in specific steps of the sulfate assimilatory pathway, to isolate and identify the corresponding gene and gene products in T. ferrooxidans. Some of the E. coli mutants that were utilized for this study are shown in Table 2.

In E. coli, a number of the genes that are involved in sulfur assimilation are located at a locus mapping at 59 minutes on the chromosome. In particular, a cluster designated cysDNCHIJ that encodes the ATP sulfurylase, APS kinase, PAPS reductase and sulfite reductase respectively, form part of an operon (Jones-Mortimer, 1973, Leyh et al., 1988).

Using DNA from pooled clones from the T. ferrooxidans genomic library we transformed JM221, an E. coli mutant with a defective ATP sulfurylase, to cysteine independence. The selection was for those plasmids that could simultaneously confer the mutant ampicillin resistance and the ability to grow on plates containing sulfate as the only sulfur source. Two such plasmids were obtained and one of them, designated pTFcys13, was chosen for further studies. A map of pTFcys13 showing the restriction sites present in the 4.7 kb T. ferrooxidans insert is shown in figure 2. The origin of the pTFcys13 insert was confirmed by a Southern Blot experiment with T. ferrooxidans DNA digested with restriction enzymes to compare sequences in the chromosome with those of pTFcys13. The results are shown in Fig.3. As expected,only those lanes in which total digested T. ferrooxidans DNA is present showed hybridization bands with the 4.7 Kb BamHI insert probe from pTFcys13 (lanes 2, 3 and 7). No homology was observed between the probe and genomic DNA from JM221(lane 11).

Since in other bacterial species the genes involved in sulfate assimilation occur as a cluster on a specific region of the chromosome (Jones-Mortimer, 1968; Leyh et al., 1988), we tested pTFcys13 for the ability to complement a number of E. coli mutants defective in the sulfur assimilatory pathway. Mutants used for this purpose included: JM246 (cysI), AT2427(cysJ), JM81A(cysC) and DG37 (cysA). In addition to JM221, only JM81A yielded recombinants in which sulfate utilization had been restored. Therefore, it was assumed that besides the cysD gene, the cysC gene, encoding the APS kinase was present on the pTFcys13 insert. The sulfite reductase gene, that in the E.coli cys operon follows the cysC gene, did not appear to be present in this insert.

In vivo complementation and growth studies

The growth properties and the levels of ATP sulfurylase in response to exogenous sulfur source were studied in the JM221 mutant carrying pTFcys13. As shown in figure 4, panel B, pTFcys13 restores JM221 the ability to grow in sulfate minimal medium. In fact, JM221/pTFcys13 can grow in this medium at the same rate as if the medium is supplemented with cysteine or reduced glutathione. In contrast, if JM221 carries the pBR322 vector only, no restoration of sulfate utilization is observed (panel A). The putative regulation of the cloned T. ferrooxidans genes has been examined by measuring the levels of ATP sulfurylase and sulfite reductase (Fry et al 1988). It was found that the levels of ATP sulfurylase activity conferred by pTFcys13 were unaffected by the sulfur source present in the growth medium. Thus, in

contrast to that of E.coli , the cloned ATP sulfurylase from T. ferrooxidans does not seem to be regulated by the level of intracellular cysteine or reduced glutathione.

Genetic organization and gene products encoded from the T. ferrooxidans cys locus.

To ascertain the genetic organization of the cysD and cysC loci present in pTFcys13 a number of deletions extending into the insert region of this plasmid were constructed and examined for their complementation pattern (Fry et al 1989). Only a deletion that extended from the HindIII site of the pBR322 vector to the HindIII site 1 kb into the insert (figure 2), was found to retain the ability to complement JM221(cysD) while becoming unable to complement JM81A (cysC). This was the first indication of a possible three-gene arrangement for this locus and suggested that the the ATP sulfurylase might be encoded by more than one gene. Evidence for this genetic organization and for the fact that two gene products are being encoded by the cysD locus was obtained by subcloning the fragment remaining in the pTFcys13ΔHindIII plasmid into the pBS$^-$ cloning vector (Stratagene, La Jolla, CA.). The resulting plasmid, pBScys13ΔHindII which lacked APS kinase-complementing activity by virtue of a deletion extending into the cysC gene, was used for in vitro protein synthesis analysis of the cysD region. The deletion plasmid was digested with a series of restriction enzymes and the resultant linear DNA fragments (figure 5) were used to direct an in vitro transcription-translation system that enables the identification of polypeptides made from a DNA template. Two unique products not present in the pBR322 control were readily identified by autoradiography: a 62 and 36 Kd polypetide respectively (indicated by arrows in Figure 6.). It was noted that the EcoRI-treated fragment gave rise to a truncated 61Kd polypeptide (lane 3). While in the SacI-digested fragment the synthesis of the 36Kd peptide was completely abolished(lane 4). By correlating the peptides obtained from the various restriction fragments from pBScys13DHindIII with the parallel complementation and deletion studies, it was possible to infer the order of the genes of this locus and permited the identification of the respective polypeptides that they encode. Thus, it was determined that the cysD gene, the first gene of this three-gene cluster, encodes the 62 Kd polypeptide (CysD product). While the cysN gene, which lies between cysD and the distal cysC gene, encodes the 36 Kd polypeptide (CysN product). This finding established the multimeric make up of the T. ferrooxidans sulfurylase. A summary depicting the gene arrangement, the corresponding peptides derived from this locus and a comparison between the analogous regions of E. coli and T. ferrooxidans is shown in figure 6.

CONCLUSIONS

Confirmation of the presence of ATP sulfurylase and APS kinase by this work supports the notion that sulfate assimilation to form cysteine and methionine operates through the cys pathway in T. ferrooxidans. Under proper growth medium conditions, T. ferrooxidans oxidizes inorganic sulfur compounds such as sulfur, tetrathionate and thiosulfate to obtain metabolic energy in the form of ATP. Both the dissimilatory and assimilatory pathway appear to have APS as an intermediate. That these two diametrically opposing reactions (which could easily lead to a futile cycle) could be occuring simultaneously has been questioned (Tuovinen et al., 1975). Kelley et al. (1976) determined that $^{35}SO_4$ was only taken up and assimilated when the growth medium for T. ferrooxidans was ferrous sulfate-based. However, these investigators partially purified an active ATP sulfurylase form both thiosulfate and ferrous sulfate grown cell whose levels did not appear to vary in response to the nature of the medium. They thus, concluded that the synthesis of ATP sulfurylase was constitutive but that it did not fulfill an assimilatory function when cells were grown on thiosulfate medium. Rigorous studies of the regulation of these genes cannot be carried out in a heterologous host such as E. coli, nevertheless, the fact that the synthesis of this enzyme, when produced in E. coli, was not succeptible to regulation by the levels of exogenous cysteine is consistent with a pattern of constitutive synthesis.

That the genes involved in the assimilation of sulfate are arranged in an operon-like structure in T. ferrooxidans was not entirely unexpected since other genes such as the nitrogen fixing (nif) genes (Pretorious et al., 1986), the glnALG genes (Barros et al., 1985) and the genes of the ribosomal operon (Venegas et al, 1988) also show this arrangement in T. ferrooxidans . It is nevertheless interesting to note the fact that organisms as phylogenetically different as E. coli and T. ferrooxidans seem to posses gene organizations so similar to one another.

Acknowledgements

This work was performed at the Lawrence Livermore National Laboratory under the auspices of the U.S. Department of Energy Contract No. W-7405-ENG-48.

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TABLE 1

Bacterial Strains Used in This Study

Strains	Genotype	Source or Reference
HB101	pro- leu thi⁻ lacY hsdR⁻ endA⁻ rpsL20 ara-14 galK2 xyl5 mtl⁻ 1 supE44 recA⁻	Bachmann and Bolivar, 1979
JM221	cysD91 pro50 his97 trp⁻ 74 argA⁻ ilvA lac⁻ gal⁻ galT47 xyl⁻ mtl⁻ mal⁻ strA⁻ tsx⁻	received as CGSC 5745 from B. Bachmann
JM246	cys153 lambda⁻ 1N(rrnD-rrnE)1	received as CGSC 4747 from B. Bachmann
AT2427	sysJ43 relA1 thi⁻ 1 lambda-spot1	received as CGSC 4502
JM96	cysH56 thr⁻ 1 leuB6 trp⁻ 1 argH1 thi⁻ 1 ara⁻ 13 laY1 gal⁻ 6 malA1 xyl⁻ 7 mtl⁻ 2 strA9 tonA2 supE44 lambda⁻ lambda. R	received as CGSC 5746 from B. Bachmann
JM81A	cysC	received as CGSC 5744 from B. Bachmann
T. ferroxidans (ATCC 218361)	wild-type	ATCC

TABLE 2

Pattern of Complementation of pTFcys13 on Various *E. coli* Mutants Defective in the Sulfate Assimilatory Pathway

Mutant		Response*
JM221	(*cys* D)	+
JM81A	(*cys* C)	+
AT2427	(*cys* J)	-
JM246	(*cys* I)	-
JM96	(*cys* H)	-
DG37	(*cysA*)	-

*A positive response denotes growth with sulfate as sulfur source.

TABLE 3

ATP Sulfurylase And Sulfite Reductase Activity In Crude Extracts of E. Coli And T. Ferrooxidans

		Specific Activity	
		ATP Sulfurylase pMole APS /min/mg	Sulfite Reductase nMole NADP /min/mg
HB101/pBR322			
1.	SULFATE	210.0	8.78
2.	CYSTEINE	<0.3	<0.10
3.	RED. GSH	1260.0	6.10
JM221/pBR322			
4.	RED. GSH	<0.3	3.57
JM221/pTFcys13			
5.	SULFATE	29.0	3.34
6.	CYSTEINE	21.6	<0.1
7.	RED. GSH	21.1	2.87
T. ferrooxidans *		3.3	1.9

Cell extracts were assayed for ATP sulfurylase activity according to the method of Hanna and Taylor (1989) and for sulfite reductase activity using the method of Yoshimoto et al. (1971). Source of the crude extract were cells grown to mid-logarithmic phase in minimal medium containing 0.7 mM of the sole sulfur source indicated above (Fry et al., 1988; Hanna and Taylor, 1989)
* Grown in Fe SO_4

CAPTIONS FOR FIGURES

Figure 1.Generalized Scheme of Microbial Sulfur Metabolism. (A) Proposed pathway for the oxidation of inorganic sulfur compounds in the thiobacilli (modified from Siegel, 1975). (B) Pathway of sulfate assimilation in enterobacteriace. The genetic loci indicated for each enzymatic step are those defined for S. typhimurium (modified form Kredich, 1987).

Figure 2. Physical Map of pTFcys13. Restriction endonuclease sites within the 4.7 KB BamHI insert (thick line) of T. ferrooxidans are shown. The direction of transcription of the b-lactamase gene of the pBR322 vector is illustrated by the arrow.

Figure 3. Southern blot analysis of pTFcys13. Total cellular DNA from T. ferrooxidans and E. coli was digested to completion with the restriction enzymes indicated and separated by electrophoresis on a 0.8% agarose gel. After transfer to nitrocellulose, the DNA was hybridize with the [^{32}P]labeled BamHI insert from pTFcys13. Lane 1,T. ferrooxidans (TF) uncut;lane 2,TF BamHI; lane 3,TF EcoRI; lane 4, TF EcoRV; lane 5, TF HindIII; lane 6, TF PstI; lane 7,TF SacI; lane 8, TF SalI; lane 9, JM221 BamHI ; lane 10, pTFcys13 BamHI.

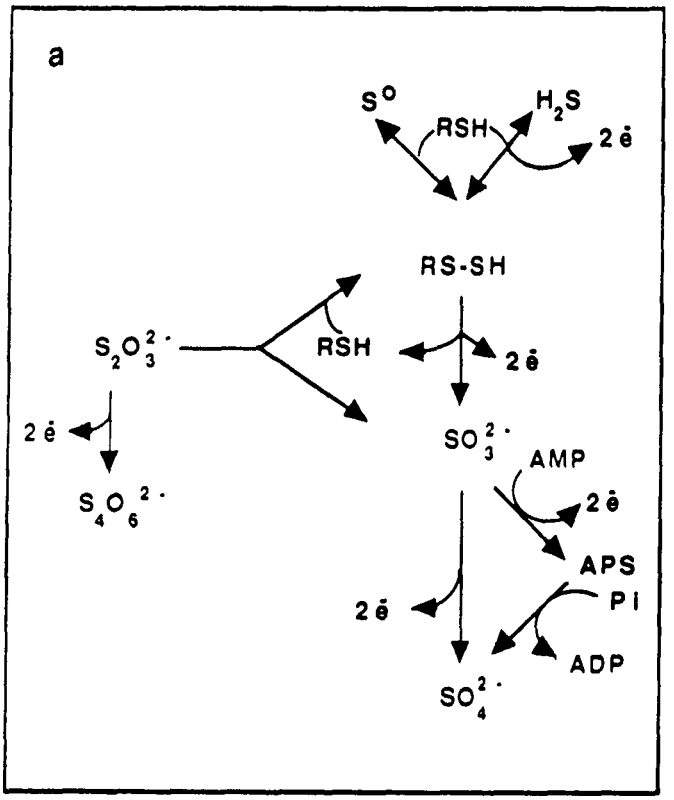
Figure 4. Growth curves of JM221/pBR322 (panel A) and JM221/pTFcys13 (panel B). Cells grown in sulfate minimal medium (•); cysteine-supplemented medium (■); and in medium supplemented with glutathione (▲).

Figure 5. Map of restriction endonuclease-generated fragments used in the synthesis of proteins in an E. coli DNA-directed cell-free system. Fragments indicated by the arrows were obtained by linearizing pBScys13DHindIII with PstI, EcorI and SacI respectively.

Figure 6. DNA-dependent cell-free synthesis of cloned proteins.The gene products labeled with [^{35}S]methionine were analyzed on SDS-polyacrylamide gel electrophoresis.DNA template was linearized with restriction enzymes as indicated. Lane 1, pBScys13ΔHindIII uncut; lane 2, pBScys13ΔHindIII/PstI; lane 3, pBScys13ΔHindIII/EcoRI; lane 4, pBScys13ΔHindIII/SacI

Figure 7. Physical map and polypeptide expression in the T. ferrooxidans cloned insert and its comparison to the analogous cloned region from E. coli (Leyh, 1988). The approximate location of the region of DNA encoding for the protein products was obtained from

experiments described in the text. The direction of transcription has not been determined but it is presumed to proceed from cysD to CysC.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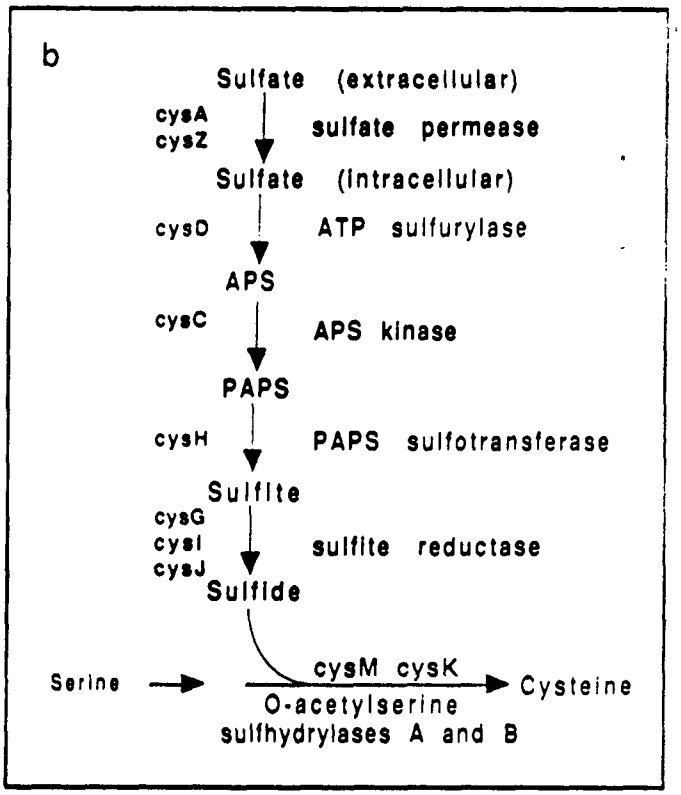
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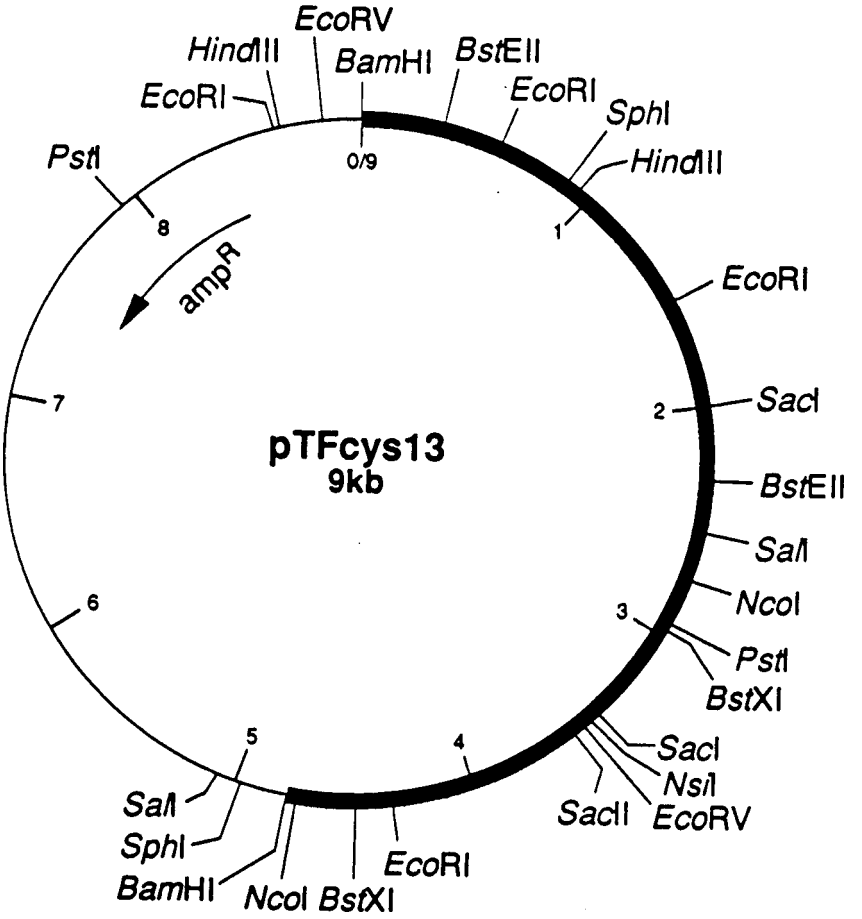
Fig. 1, Page 3



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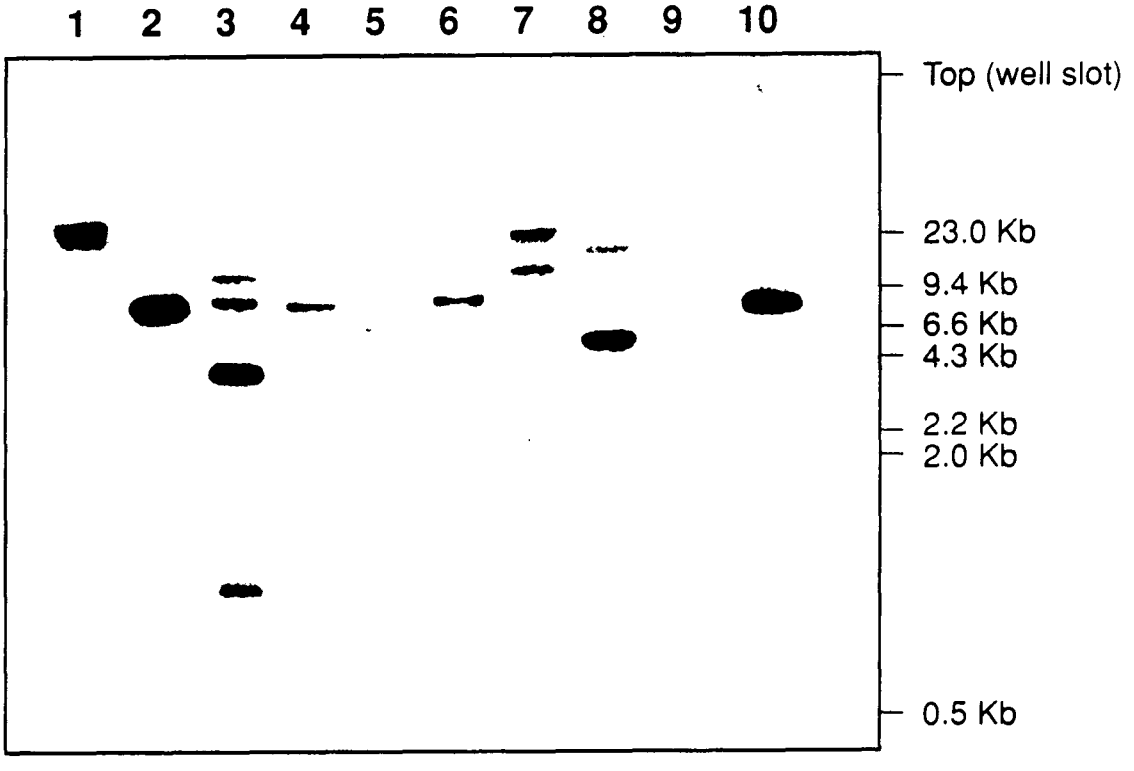
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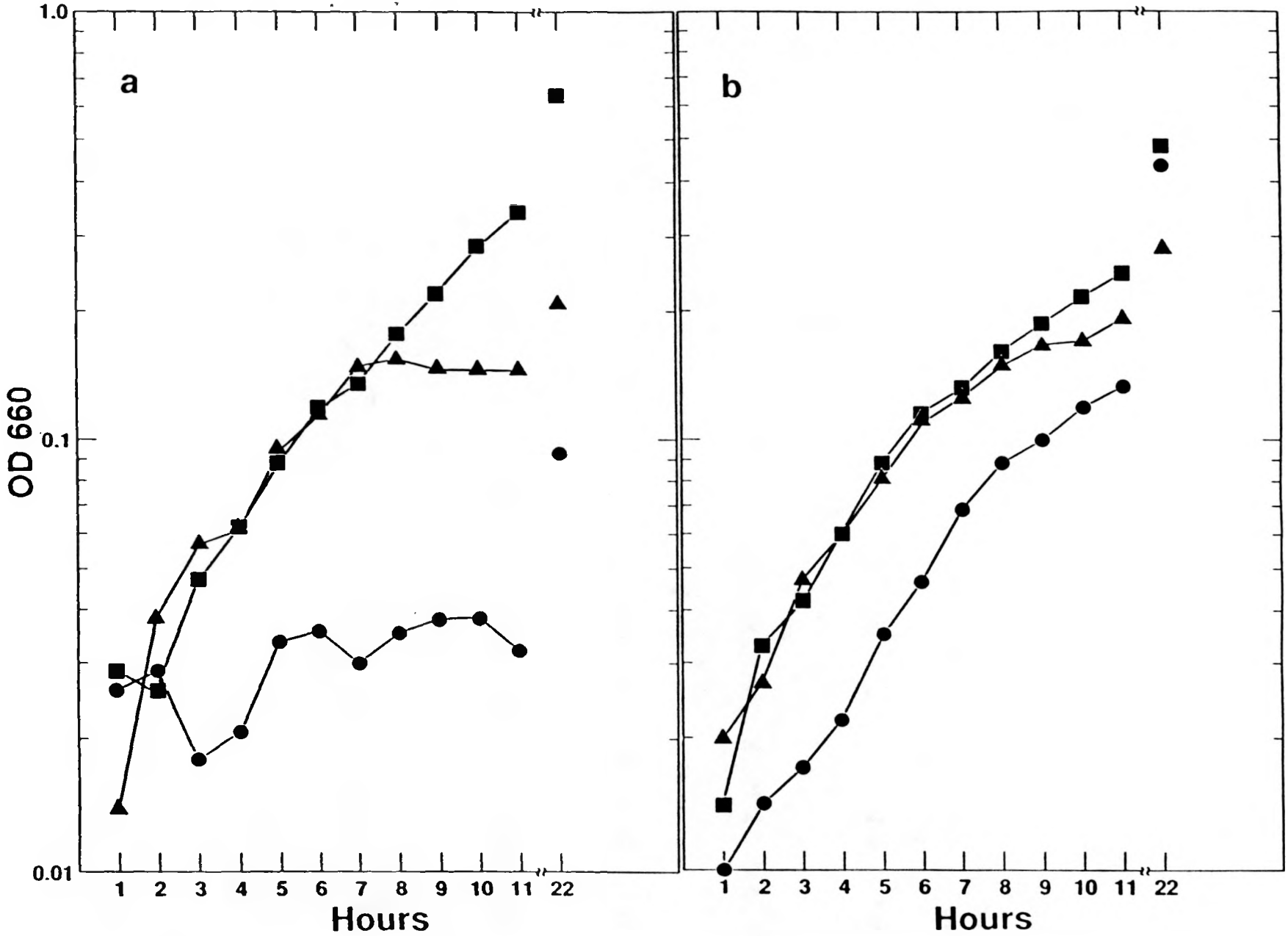
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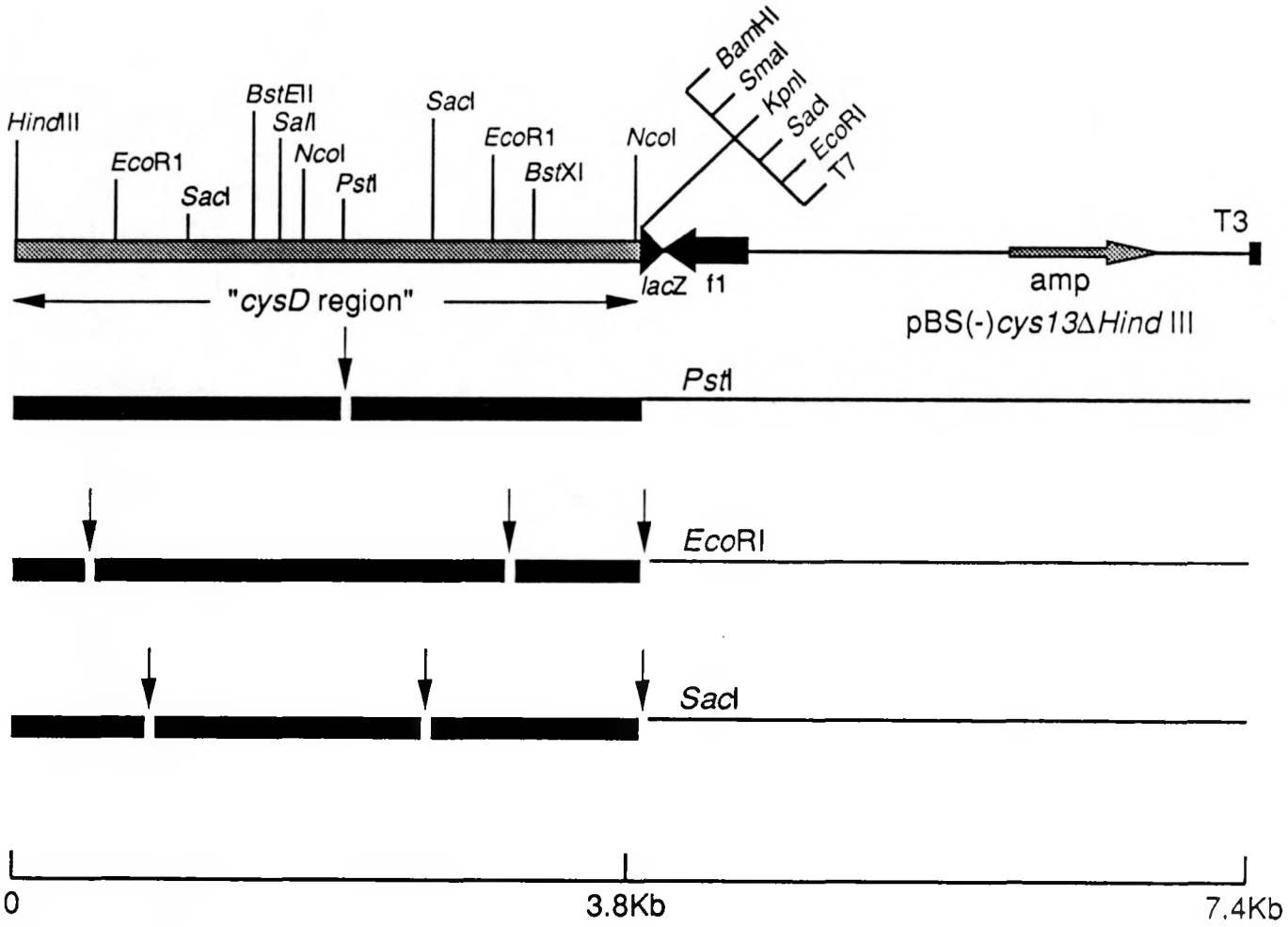
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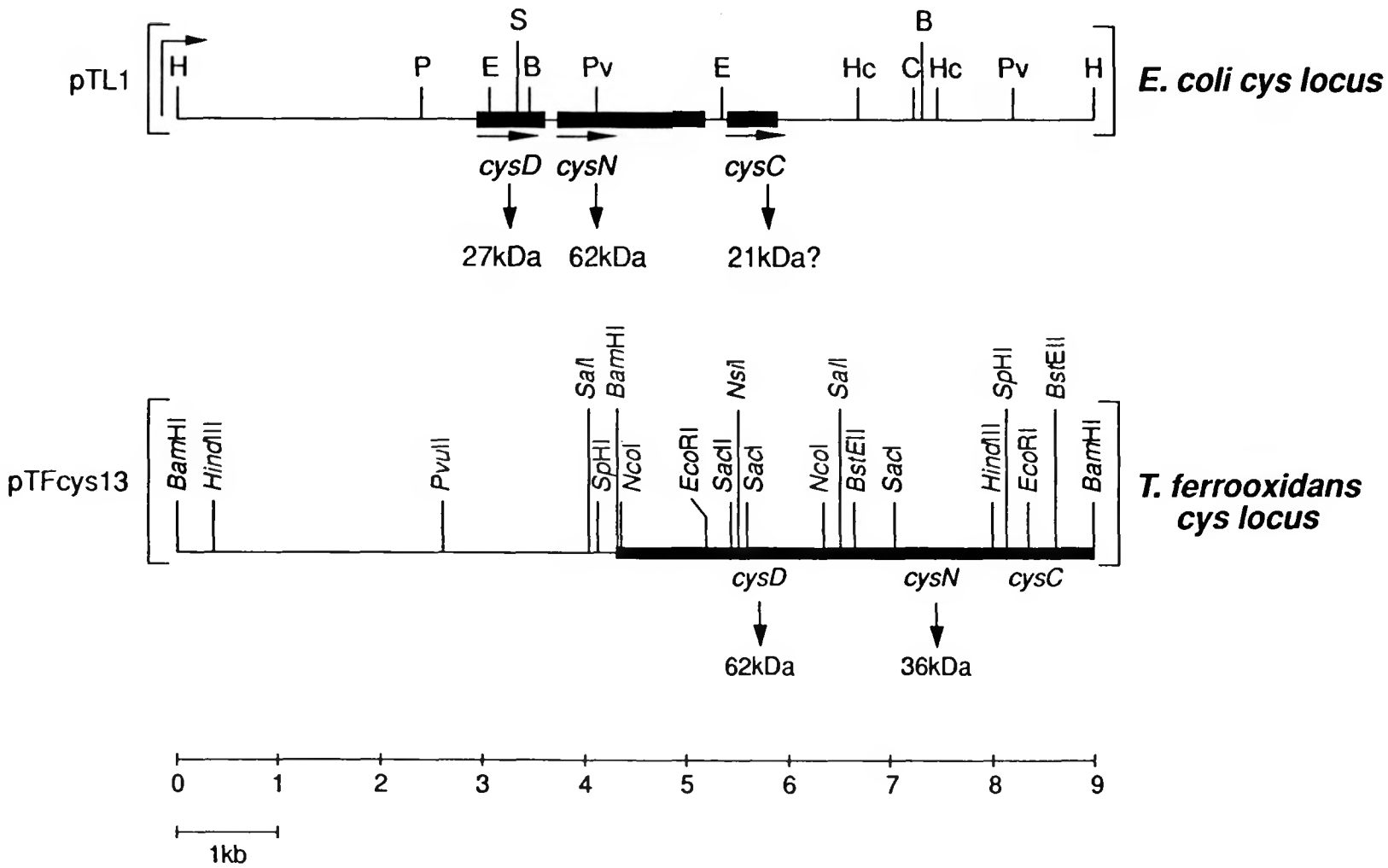

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