JOURNAL OF BACTERIOLOGY, Feb. 1991, p. 1088–1095
0021-9193/91/031088-08$02.00/0

Vol. 173, No. 3

Metronidazole Activation and Isolation of *Clostridium acetobutylicum* Electron Transport Genes

JOSEPH D. SANTANGELO, DAVID T. JONES,† AND DAVID R. WOODS*

Department of Microbiology, University of Cape Town, Rondebosch 7700, South Africa

Received 13 August 1990/Accepted 21 November 1990

An *Escherichia coli* F19 *recA*, nitrate reductase-deficient mutant was constructed by transposon mutagenesis and shown to be resistant to metronidazole. This mutant was a most suitable host for the isolation of *Clostridium acetobutylicum* genes on recombinant plasmids, which activated metronidazole and rendered the *E. coli* F19 strain sensitive to metronidazole. Twenty-five *E. coli* F19 clones containing different recombinant plasmids were isolated and classified into five groups on the basis of their sensitivity to metronidazole. The clones were tested for nitrate reductase, pyruvate-ferredoxin oxidoreductase, and hydrogenase activities. DNA hybridization and restriction endonuclease mapping revealed that four of the *C. acetobutylicum* insert DNA fragments on recombinant plasmids were linked in an 11.1-kb chromosomal fragment. DNA sequencing and amino acid homology studies indicated that this DNA fragment contained a flavodoxin gene which encoded a protein of 160 amino acids that activated metronidazole and made the *E. coli* F19 mutant very sensitive to metronidazole. The flavodoxin and hydrogenase genes which are involved in electron transfer systems were linked on the 11.1-kb DNA fragment from *C. acetobutylicum*.

Metronidazole, 5-nitroimidazole, is a widely used drug with a broad spectrum of antimicrobial activity. It is effective against both gram-positive and gram-negative bacteria, protozoa, and a few helminths (11). Although the spectrum is wide, anaerobic or microaerophilic conditions are required for activation of this compound. It is generally accepted that reduction of the nitro group is essential for the formation of a toxic derivative or derivatives from this relatively inert compound. There have been conflicting reports as to what proteins or enzymes in the clostridia are responsible for the reduction of metronidazole. Edwards and Mathison (12) reported that reduced ferredoxin could transfer electrons directly to metronidazole, forming the reduced toxic derivatives. This theory is supported by the hydrogenase-linked assay system developed by Chen and Blanchard (4) for measuring ferredoxin and flavodoxin, with metronidazole as the indicator compound. These authors report that hydrogenase transfers electrons from hydrogen gas to ferredoxin or flavodoxin, which then transfers electrons to metronidazole, forming the reduced toxic intermediates. It has also been reported that metronidazole is reduced enzymatically by the action of pyruvate-ferredoxin oxidoreductase (32). The most recent theory has been put forward by Church et al. (6), who have shown that metronidazole is reduced by hydrogenase 1 of *Clostridium pasteurianum*.

The bacteriocidal toxic derivative of metronidazole has been shown to cause DNA damage (23). Chrystal et al. (5) proposed a model relating metronidazole metabolism to bacteriocidal action. Metronidazole is reduced to its active form, M*, which can react with DNA and thus cause DNA damage. If the rate of DNA damage is greater than the rate of repair, cell death occurs. Therefore, a cell with an efficient DNA repair mechanism is able to tolerate higher levels of M* than a cell with a less efficient DNA repair system. Since the rate of formation of M* is directly proportional to the rate of DNA damage, two mechanisms of resistance to metronidazole exist. One mechanism involves an efficient DNA repair system, and the other involves the lack of the ability to reduce metronidazole to its active intermediate, M*. In *Escherichia coli*, anaerobic conditions are required for the reduction of metronidazole to its toxic derivative. It has been demonstrated that DNA repair mutants of *E. coli* were killed more efficiently by metronidazole than were their respective parental strains. However, if these DNA repair mutants also lacked the ability to reduce nitrate and chlorate, they were no more susceptible to metronidazole than were their wild-type parents (47). This indicates that the nitrate and chlorate reductase systems of *E. coli* are responsible for reducing metronidazole to its active form.

An understanding of the mechanisms involved in activating metronidazole is important for the development of improved analogs for use in medicine. To achieve this, we developed a negative selection system for the cloning of genes controlling the activation of metronidazole in *E. coli*. An additional advantage of this system is that it enabled us to clone genes involved in the electron transport system of *Clostridium acetobutylicum*. These genes are important in the understanding and the future manipulation of solvent pathways in the industrially important *C. acetobutylicum* strain (19).

The negative selection system involved the construction of a nitrate reductase-deficient, DNA repair-deficient mutant of *E. coli*. This mutant is highly sensitive to the reduced toxic intermediate of metronidazole because of its deficient DNA repair system, yet it is unable to form the toxic compound because of its diminished nitrate reductase activity. By using this system, 26 clones showing increased sensitivity to metronidazole were isolated and characterized.

MATERIALS AND METHODS

Materials. Restriction endonucleases were obtained from several sources (Amersham International, Amersham, United Kingdom; Anglian Biotec, Essex, United Kingdom; Boehringer Mannheim Biochemicals GmbH, Mannheim,

* Corresponding author.
† Present address: Department of Microbiology, Medical School, University of Otago, Dunedin, New Zealand.

Germany; New England BioLabs, Inc., Beverly, Mass.; and Promega Corp., Madison, Wis.) and were used as specified by the manufacturers. The enzymes exonuclease III, S1 nuclease, and PolIk were obtained from Boehringer Mannheim Biochemicals. α-^{32}P- and α-^{35}S-labeled nucleotides were obtained from Amersham International plc. Metronidazole was obtained from the Sigma Chemical Co., St. Louis, Mo. All other chemicals were analytical grade and were purchased from various local suppliers.

Bacterial strains and plasmids. *C. acetobutylicum* P262 (18) was used as the source of DNA. The nitrate reductase-deficient *E. coli* strain F19 was derived from the *recA E. coli* strain CC118 [*araD139*Δ(*ara leu*)*7697*Δ*lacX74 phoA*Δ*20 galE galK thi rpsE rpoB argE*(Am) *recA1*] (29) by transposon mutagenesis. For hydrogenase assays, plasmids were transformed into the *E. coli* SE53 *hydA* mutant which was derived from *E. coli* Puig426 (26). Phage λ*b221rex*::Tn*phoA* *c*I857 *P*am3 was a gift from C. Manoil (14). The positive selection plasmid vector pEcoR251 was a gift from M. Zabeau, Biotechnology Business Development, Ghent, Belgium, and has been described previously (49).

Media. *C. acetobutylicum* P262 was grown under strict anaerobic conditions in the *Clostridium* basal medium of O'Brien and Morris (34) as described by Allcock et al. (1). *E. coli* was grown aerobically in Luria broth (LB) (28). Anaerobic growth of *E. coli* was carried out in an anaerobic glove box (Forma Scientific Inc., Marietta, Ohio). To support growth of *E. coli* via anaerobic respiration or fermentation, bacteria were grown either in LB supplemented with 0.2% (wt/vol) $NaNO_3$ and 0.1% (wt/vol) glucose or in the complex medium (pH 8.0) described by Clark and Cronan (7) supplemented with 1% (wt/vol) glucose.

Determination of MIC. An aerobically grown overnight culture of *E. coli* was diluted 10^{-4} with sterile water, and 100 μl was spread onto prereduced agar medium containing various concentrations of antibiotic. Unless otherwise specified, the medium used for MIC determination was LB supplemented with 0.2% $NaNO_3$ and 0.1% glucose and incubated anaerobically. The MIC was considered to be the amount of antibiotic (per milliliter) that totally inhibited growth.

Preparation of DNA. Plasmid DNA was prepared by the alkali-hydrolysis method of Ish-Horowicz and Burke (16). *C. acetobutylicum* chromosomal DNA was prepared by the method of Marmur (30), which was modified (49) to overcome the high nuclease activity exhibited by *C. acetobutylicum* (43).

Transposon mutagenesis. Tn*phoA* is a derivative of Tn*5* which contains the structural gene for *E. coli* alkaline phosphatase (*phoA*) cloned into the transposon (29). The alkaline phosphatase portion of Tn*phoA* was not needed for the desired *E. coli* chromosomal mutation, and hence Tn*phoA* was used instead of Tn*5* merely because it was readily available. Transposon insertions of Tn*phoA* into the *E. coli* CC118 chromosome were isolated by using an adaptation of the protocol of Gutierrez et al. (14), as described by Scholle et al. (38). To select for metronidazole-resistant mutants, the cells were plated on LB that was supplemented with 0.2% $NaNO_3$ and 0.1% glucose and that contained 30 μg of kanamycin per ml and 30 μg of metronidazole per ml. These plates were incubated anaerobically at 34°C, and the resulting metronidazole-resistant colonies were tested for *recA* reversion and nitrate reductase activity.

Cloning of *C. acetobutylicum* genes that cause an increased sensitivity to metronidazole. Competent *E. coli* F19 cells were transformed with prepared pEcoR251 plasmid pools which have been described previously (49). Recombinant pEcoR251 plasmids were selected on LB agar containing ampicillin (100 μg/ml). Colonies were duplicated onto LB agar that was supplemented with 0.2% $NaNO_3$ and 0.1% glucose and that contained ampicillin (100 μg/ml) or ampicillin plus metronidazole (30 μg/ml). These plates were incubated anaerobically at 34°C for 24 h, and colonies that were resistant to ampicillin and sensitive to metronidazole were chosen for further study.

Preparation of cell extracts. Cell extracts of *E. coli* were prepared from overnight cultures (400 ml) grown anaerobically. *E. coli* cells containing recombinant plasmids were grown in media supplemented with ampicillin. Cell extracts of *C. acetobutylicum* were prepared from exponential-phase cells grown anaerobically in *Clostridium* basal medium. Cell extracts were prepared under anaerobic conditions by the procedure of Clark and Cronan (7) and stored at −70°C to preserve enzyme activity. Protein concentrations in the extracts were determined by the biuret method as described by Gornall et al. (13).

Enzyme assays. Pyruvate-ferredoxin oxidoreductase was assayed by the method of Wahl and Orme-Johnson (45). Hydrogenase activity was assayed by the methods of Church et al. (6). Nitrate reductase activity was assayed by determining the conversion of *p*-nitrobenzoic acid to *p*-aminobenzoic acid by the method of Bratton and Marshall (3). *recA* mutations were confirmed by a simple UV dose experiment as described by Maniatis et al. (28).

Preparation of radiolabeled probe DNA. Plasmids containing *C. acetobutylicum* DNA inserts or isolated fragments were nick translated with [α-^{32}P]dATP by using the nick translation kit of Amersham International (code N.5500). Nick-translated probes were prepared according to the manufacturer's instructions.

DNA hybridization. Plasmid DNA and *C. acetobutylicum* chromosomal DNA were digested to completion with the appropriate endonucleases, and the resulting fragments were fractionated by electrophoresis in either 0.8 or 1.2% (wt/vol) agarose gels in Tris-acetate buffer, as appropriate. The DNA was transferred monodirectionally to a Hybond N^+ nylon membrane (Amersham International) according to the manufacturer's instructions. The radiolabeled plasmids described above were used to probe these membranes (39).

Exonuclease III digestion. By using restriction enzyme deletion analysis of plasmid pMET13A, it was determined that the area responsible for metronidazole sensitivity was contained on the 2-kb *Eco*RI-*Eco*RV fragment (see Fig. 2). This fragment of pMET13A was subcloned into the *Eco*RI-*Hin*cII sites of Bluescript plasmid KS (Stratagene, San Diego, Calif.). Progressive deletions of the subclone from both the 5′ and 3′ ends of the insert were generated by unidirectionally digesting *Bst*XI-*Bam*HI and *Kpn*I-*Acc*I fragments with exonuclease III (15). The deletions were transformed into *E. coli* LK111 (48), and transformants were selected on LB agar containing ampicillin (100 μg/ml).

Nucleotide sequencing. The nucleotide sequences of both strands of the pMET13A active region were determined by using overlapping DNA fragments generated by exonuclease III digestion as described above. Nucleotide sequencing was carried out by the dideoxynucleotide triphosphate chain termination method developed by Sanger et al. (37), according to the protocol outlined by Tabor and Richardson (40), with the Sequenase DNA sequencing kit (US Biochemical Corporation, Cleveland, Ohio). The nucleotide and deduced amino acid sequences were analyzed on a VAX 6000-330

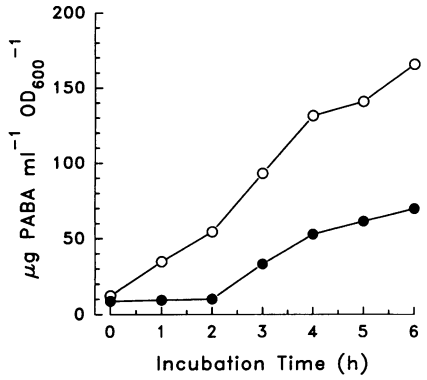

FIG. 1. Nitrate reductase activity of *E. coli* F19 (●) compared with that of its parent strain, *E. coli* CC118 (○). Nitrate reductase activity was determined by incubating cells with *p*-nitrobenzoic acid and measuring the resulting *p*-aminobenzoic acid (PABA) concentration with respect to time. OD_{600}, Optical density at 600 nm.

computer by using the Genetics Computer Group Inc. suite of sequence analysis programs (8).

Nucleotide sequence accession number. The nucleotide sequence of the pMET13A active region has been submitted to the GenBank DNA sequence data base and has been assigned the accession number M36770.

RESULTS

Transposon mutagenesis. *E. coli* CC118 was a suitable strain for transposon mutagenesis since it has a stable *recA* mutation, is not a λ-lysogen, is readily transformable, and is a proven host for Tn*phoA* (29). Following transposon mutagenesis, 222 metronidazole-resistant mutants were isolated. These mutants were tested for stability of mutation, *recA* reversion, and nitrate reductase activity. Of the 222 mutants, 10 were not stable and were unable to grow on metronidazole after being subcultured. A further 29 mutants were *recA* revertants, while 50 did not show reduced levels of nitrate reductase. The remaining 133 mutants all showed reduced levels of nitrate reductase. Mutant F19 consistently showed the lowest level of nitrate reductase activity, and the nitrate reductase level of F19 was compared with that of its parent strain, CC118 (Fig. 1). Mutant F19 was therefore chosen for screening the *C. acetobutylicum* gene bank for genes which cause an increase in sensitivity to metronidazole.

Screening of the *C. acetobutylicum* gene library. *E. coli* F19 was used as the host to screen the *C. acetobutylicum* gene bank for genes conferring metronidazole sensitivity. Although this mutant was very sensitive to the reduced toxic derivative(s) of metronidazole because of a *recA* mutation, it was resistant to metronidazole since it lacked the *E. coli* nitrate reductase system involved in the reduction of metronidazole to its toxic derivative(s). The screening of the *C. acetobutylicum* gene library in *E. coli* F19 selected for genes that complement the metronidazole-reducing ability which is lost in this mutant. Twenty-six stable clones that showed an increased sensitivity to metronidazole were isolated. Plasmid involvement in metronidazole sensitivity was confirmed by retransformation experiments with plasmids isolated from the 26 clones. Plasmids from each clone that were retransformed into *E. coli* F19 produced similar numbers of ampicillin-resistant and metronidazole-sensitive transformants. The clones were classified into five groups (I to V) on the basis of their sensitivity to metronidazole; class V was the most sensitive group, and class I was the least sensitive group. Of the 26 clones, 12 were class I isolates, 3 were class II isolates, 3 were class III isolates, 3 were class IV isolates, and 5 were class V isolates. Under conditions of anaerobic respiration, the MICs for classes I, II, III, IV, and V were 20, 15, 12, 10, and <5 μg of metronidazole per ml, respectively. Likewise, under conditions of anaerobic fermentation, the MICs for classes I, II, III, IV, and V were 6, 5, 4, 3, and <1 μg of metronidazole per ml, respectively. The MIC of metronidazole for *E. coli* F19 was 35 μg/ml when F19 was grown under conditions of anaerobic respiration and 15 μg/ml when it was grown under conditions of anaerobic fermentation. The MICs for *E. coli* CC118, the parent strain of *E. coli* F19, were identical to the MICs for the class I isolates.

Tests for enzyme activity. Since it has been reported that clostridial enzymes, pyruvate-ferredoxin oxidoreductase (32), and hydrogenase (6) may be responsible for the reduction of metronidazole, cell extracts of all 26 clones were tested for these enzymes. Since the enzyme responsible for the reduction of metronidazole in *E. coli* is nitrate reductase, the 26 clones were also tested for nitrate reductase activity. No significant enzyme activity was detected for either nitrate reductase or pyruvate-ferredoxin oxidoreductase in any of the clones. To test for hydrogenase activity, plasmids were transformed into *E. coli* SE53, which is a *hydA* mutant. Cell extracts prepared from the parent of *E. coli* SE53, *E. coli* Puig426, were used as a positive control, along with cell extracts prepared from *C. acetobutylicum*. *E. coli* SE53 containing plasmid pMET15B2 was the only isolate that tested positive for hydrogenase activity. The activity (in microliters of H_2 per milligram of protein per min) of crude extracts of *E. coli* SE53(pMET15B2) was 1.98, approximately half the activity of *E. coli* Puig426(pBR322) extracts (4.04) and only 7% of the activity of *C. acetobutylicum* P262 extracts (28.18). All the other clones showed hydrogenase levels comparable to that of the negative control (0.22 μl/mg/min), which was *E. coli* SE53 transformed with pBR322.

DNA hybridization studies. To determine the relatedness of the 26 clones, DNA hybridization studies were performed. Each plasmid, as well as pEcoR251 with no insert, was nick translated, yielding a total of 27 radiolabeled probes. Unlabeled plasmids were digested to completion with *Hin*FI endonuclease, fractionated by electrophoresis, and blotted onto nylon membranes. Because *Hin*FI has a 4-bp recognition sequence, it was assumed that at least one fragment consisting of only *C. acetobutylicum* insert DNA would be generated for each plasmid. The plasmid probes yielded autoradiographs showing the extent of cross hybridization between clones (Table 1). As determined by the restriction endonuclease digests and these DNA hybridization studies, only isolates pMET4B and pMET5E contained identical inserts. Therefore, of the 26 clones, 25 individual cloning events were represented. Ten of the clones hybridized only with themselves, 12 hybridized with 1 other clone, and 4 hybridized with 2 or more clones. The clones pMET13A, pMET15B2, pMET030, and pMET190 make up the most intriguing group, i.e., those clones that hybridized with two or more other clones. It is interesting that, with the exception of clone pMET15B2, all of the isolates in this group have a class V sensitivity to metronidazole. This may indicate that clones pMET13A, pMET030, and pMET190 all contain the same active region from the *C. acetobutylicum* chromosome.

Restriction endonuclease mapping and deletion studies. The

TABLE 1. Results of cross-hybridization studies of 26 plasmids isolated from the *C. acetobutylicum* gene library[a]

Plasmid probe	Sensitivity class	Insert size (kb)	Plasmid(s) cross hybridized with probe
pMET1A	I	5.15	
pMET2D	II	4.90	pMET14B
pMET4A	II	1.20	
pMET4B	IV	4.30	pMET5E
pMET5E	IV	4.30	pMET4B
pMET7A	I	6.35	
pMET7C	I	6.05	pMET140
pMET8E	II	5.35	pMET13B
pMET9A	I	5.20	
pMET9D	III	4.75	
pMET10B	IV	2.25	pMET040
pMET10D	III	6.75	
pMET11B	V	7.60	
pMET13A	V	6.65	pMET15B2, pMET030, pMET190
pMET13B	I	6.10	pMET8E
pMET13C1	V	4.80	
pMET13C2	I	5.65	
pMET14B	III	9.90	pMET2D
pMET15B2	I	1.00	pMET13A, pMET030
pMET15D	I	9.60	pMET130
pMET020	I	6.25	
pMET030	V	7.05	pMET13A, pMET15B2, pMET190
pMET040	I	3.35	pMET10B
pMET130	I	4.75	pMET15D
pMET140	I	5.25	pMET7C
pMET190	V	7.95	pMET13A, pMET030

[a] All plasmids were composed of the pEcoR251 vector containing *C. acetobutylicum* insert DNA.

relationship between clones pMET13A, pMET15B2, pMET030, and pMET190 was determined by restriction endonuclease mapping. The restriction map of the insert DNA of these four clones is shown in Fig. 2. As can be seen from these restriction maps, insert DNA of clone pMET15B2 is completely contained within clones pMET13A and pMET030. Furthermore, insert DNA of clone pMET15B2 overlaps slightly with clone pMET190; however, this overlap was not identified in the hybridization studies. This lack of identification is most likely due to the experimental design of the hybridization studies; the probes used in the studies contained insert DNA as well as pEcoR251 vector DNA. The small overlap from clone pMET190 was most likely linked to pEcoR251 vector DNA and was therefore disregarded, as it hybridized with the pEcoR251 (vector only) probe. Although pMET030 and pMET13A contained the same region as pMET15B2, which showed hydrogenase activity, these two plasmids did not express hydrogenase activity in *E. coli* SE53.

By the use of restriction endonuclease deletions of the insert DNA of clone pMET13A, the region responsible for class V sensitivity to metronidazole was localized to a 2-kb *Eco*RI-*Eco*RV fragment (Fig. 2). To verify that this active region was contained in clones pMET030 and pMET190 and that this DNA originated from *C. acetobutylicum* P262, Southern blot analysis was performed, with the *Sty*I-*Eco*RV fragment from clone pMET13A used as a probe (Fig. 3). Chromosomal DNA isolated from *C. acetobutylicum*, as well as plasmid DNA isolated from clones pMET11B, pMET13A, pMET13C1, pMET030, and pMET190, was digested with *Sty*I and *Eco*RV, and the resulting fragments were separated by electrophoresis. The *Sty*I-*Eco*RV, and the resulting fragments were separated by electrophoresis. The *Sty*I-*Eco*RV fragment hybridized to *Sty*I-*Eco*RV fragments of identical size from clones pMET13A, pMET030, and pMET190 as well as to a *Sty*I-*Eco*RV fragment of identical size from *C. acetobutylicum* chromosomal DNA. This hybridization indicates that the region of DNA responsible for class V sensitivity in clone pMET13A is contained within clones pMET030 and pMET190, and it also verifies that this DNA originated from the *C. acetobutylicum* chromosome. Plasmids pMET11B and pMET13C1 were included in this hybridization study because they also demonstrate class V sensitivity to metronidazole. Since the active region from pMET13A did not hybridize with either pMET11B or pMET13C1, the class V sensitivity to metronidazole associated with pMET11B and pMET13C1 is unique and separate from that associated with clones pMET13A, pMET030, and pMET190.

Nucleotide sequence of the pMET13A active region. Nucleotide sequencing of the *Eco*RI-*Eco*RV active region of pMET13A showed that two partial open reading frames and

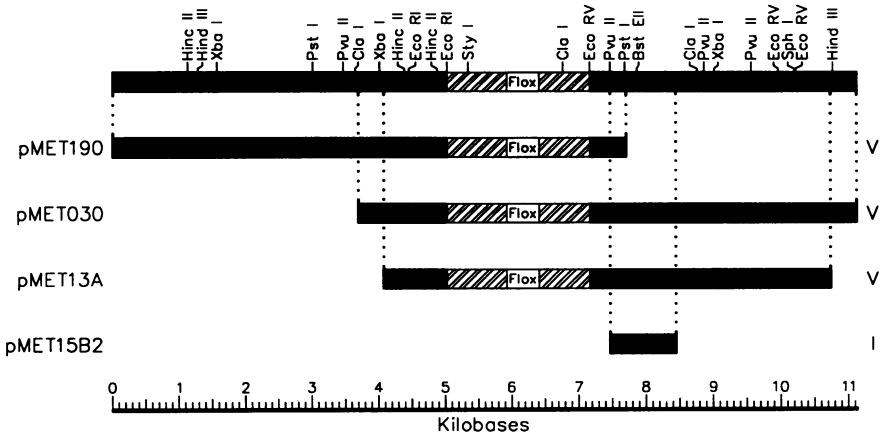

FIG. 2. Restriction endonuclease map showing the relationship between clones pMET13A, pMET15B2, pMET030, and pMET190. The arbitrary metronidazole sensitivity class is shown to the right of each clone map. The hatched region represents the area for which the DNA sequence has been determined, including the area representing the flavodoxin gene (Flox).

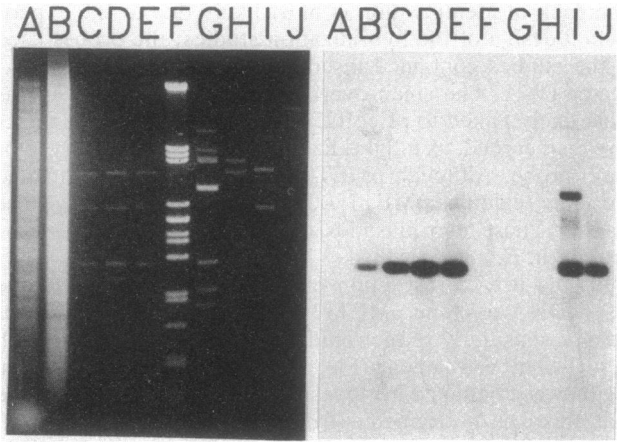

FIG. 3. Southern blot analysis of five clones that show class V sensitivity to metronidazole. The agarose gel (left panel) and the corresponding autoradiograph (right panel) are shown. The 1.6-kb *Sty*I-*Eco*RV fragment from pMET13A was nick translated and used to probe against *Sty*I-*Eco*RV-digested DNA from the *E. coli* chromosome (lanes A), the *C. acetobutylicum* chromosome (lanes B), pMET13A (lanes C, D, and E), pMET11B (lanes G), pMET13C1 (lanes H), pMET030 (lanes I), and pMET190 (lanes J). Lanes F contain *Pst*I-digested λ DNA to serve as size standards.

one complete open reading frame were present. The first partial open reading frame (159 amino acids) was truncated by the *Eco*RI site, and similarly the second partial open reading frame (164 amino acids) was truncated by the *Eco*RV site. Further nucleotide sequencing is necessary to complete these open reading frames, and therefore they will not be discussed in this report. The complete open reading frame (160 amino acids), which lies between the two truncated open reading frames, is preceded by a 230-nucleotide intergenic region and followed by an 88-nucleotide intergenic region (Fig. 4). The ATG start codon is preceded by a putative ribosome binding site (AGGAGG) 9 bp upstream of the ATG codon. A putative −35 −10 consensus promoter sequence was located between nucleotides 124 and 160 (Fig. 4).

Amino acid similarity. The deduced amino acid sequence of the complete open reading frame was used to search the GenBank, EMBL, SWISS-protein, NBRF-nucleic, and NBRF-protein data bases by using the FASTA and TFASTA computer programs described by Pearson and Lipman (36). The sequence was most similar to the flavodoxin sequences from *Azotobacter vinelandii* (2), *Clostridium* strain MP (41), *Anabaena variabilis* (27), and *Klebsiella pneumoniae* (9). GAP, the alignment program based on the method of Needleman and Wunsch (33), was used to compare this complete open reading frame with these flavodoxin sequences. The amino acid sequence of *A. vinelandii* flavodoxin had 29% identity and 55% similarity to the complete open reading frame. The amino acid sequences of the *Clostridium* strain MP, *Anabaena variabilis*, and *K. pneumoniae* flavodoxins had 29, 26, and 24% identity and 51, 55, and 56% similarity to the complete open reading frame, respectively. On the basis of the alignments with these and other flavodoxins, as well as of the fact that Chen and Blanchard (4) have reported that reduced flavodoxin can transfer electrons to metronidazole, we presumptively identify this gene as one coding for a flavodoxin from *C. acetobutylicum* P262.

To verify that this flavodoxin gene was responsible for making *E. coli* F19 sensitive to metronidazole, the progressive exonuclease III shortenings used for the nucleotide sequencing were tested for their ability to make *E. coli* F19 sensitive to metronidazole. Loss of this ability was obtained with upstream shortenings after position 195 (Fig. 4), which is 46 bp upstream from the ATG start codon. At the opposite end of the open reading frame, deletion of the last four amino acids resulted in the loss of the ability to make *E. coli* F19 sensitive to metronidazole. There was therefore a direct correlation between the flavodoxin open reading frame and the ability to activate metronidazole.

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        10                  30                  50                  70                  90                 110
TAGTAGATTATAGAATGAGTAAAAATAAATTTGAATTTTAAATTATATAGTTACTGAGTATTAATATTCATGATAAAAAAAAATGATAGCAAAAACTAAAATGTTACTATAAATTATTTTG

       130                 150                 170                 190                 210                 230
GTATTAACAAAAGCATTGTGCATGATGTGGAATTATTAATTCCAAGATGATACAATAAGTCTAAAATATACAATTTAATTATTTTAAACAATATAGTTTTTAATTAGGAGGATTTTATCA
  -35                                 -10                                                                 SD

241 ATG AAA ATA TCA ATA CTA TAC AGT AGC AAG ACA GGA AAA ACC GAA AGA GTT GCC AAG TTA ATT GAA GAA GGT GTG AAG AGG TCT GGA AAT
     M   K   I   S   I   L   Y   S   S   K   T   G   K   T   E   R   V   A   K   L   I   E   E   G   V   K   R   S   G   N

331 ATT GAA GTA AAA ACT ATG AAT TTA GAT GCA GTT GAT AAA AAG TTC TTA CAA GAA TCA GAA GGA ATT ATT TTT GGA ACA CCA ACT TAT TAT
     I   E   V   K   T   M   N   L   D   A   V   D   K   K   F   L   Q   E   S   E   G   I   I   F   G   T   P   T   Y   Y

421 GCC AAC ATA TCA TGG GAA ATG AAA AAA TGG ATA GAT GAA TCA TCA GAA TTT AAT TTA GAA GGA AAA CTT GGA GCA GCA TTT TCA ACA GCT
     A   N   I   S   W   E   M   K   K   W   I   D   E   S   S   E   F   N   L   E   G   K   L   G   A   A   F   S   T   A

511 AAC TCT ATT GCT GGT GGT TCA GAT ATA GCA TTA CTT ACA ATT TTA AAT CAT CTT ATG GTA AAA GGA ATG TTA GTA TAT TCA GGT GGA GTT
     N   S   I   A   G   G   S   D   I   A   L   L   T   I   L   N   H   L   M   V   K   G   M   L   V   Y   S   G   G   V

601 GCT TTT GGA AAA CCA AAG ACT CAT TTA GGA TAT GTG CAT ATA AAT GAA ATA CAA GAA AAT GAA GAT GAA AAT GCG AGA ATA TTT GGT GAA
     A   F   G   K   P   K   T   H   L   G   Y   V   H   I   N   E   I   Q   E   N   E   D   E   N   A   R   I   F   G   E

691 AGA ATT GCA AAC AAA GTC AAA CAA ATA TTT TAA TGTAAAAATGTATATATAAATTGATTAATAATTTAAAAATATACAATATTATAATATTGAGAGCGAAGTTTATAA
     R   I   A   N   K   V   K   Q   I   F   *
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FIG. 4. Nucleotide sequence of the flavodoxin gene and flanking regions from *C. acetobutylicum*. The deduced amino acid sequence is given in single-letter code from nucleotide positions 241 to 720 (160 amino acid residues). The −10 and −35 regions of the putative promoter are boldface and underlined. The putative ribosome binding site (SD) is boldface, underlined, and overlined.

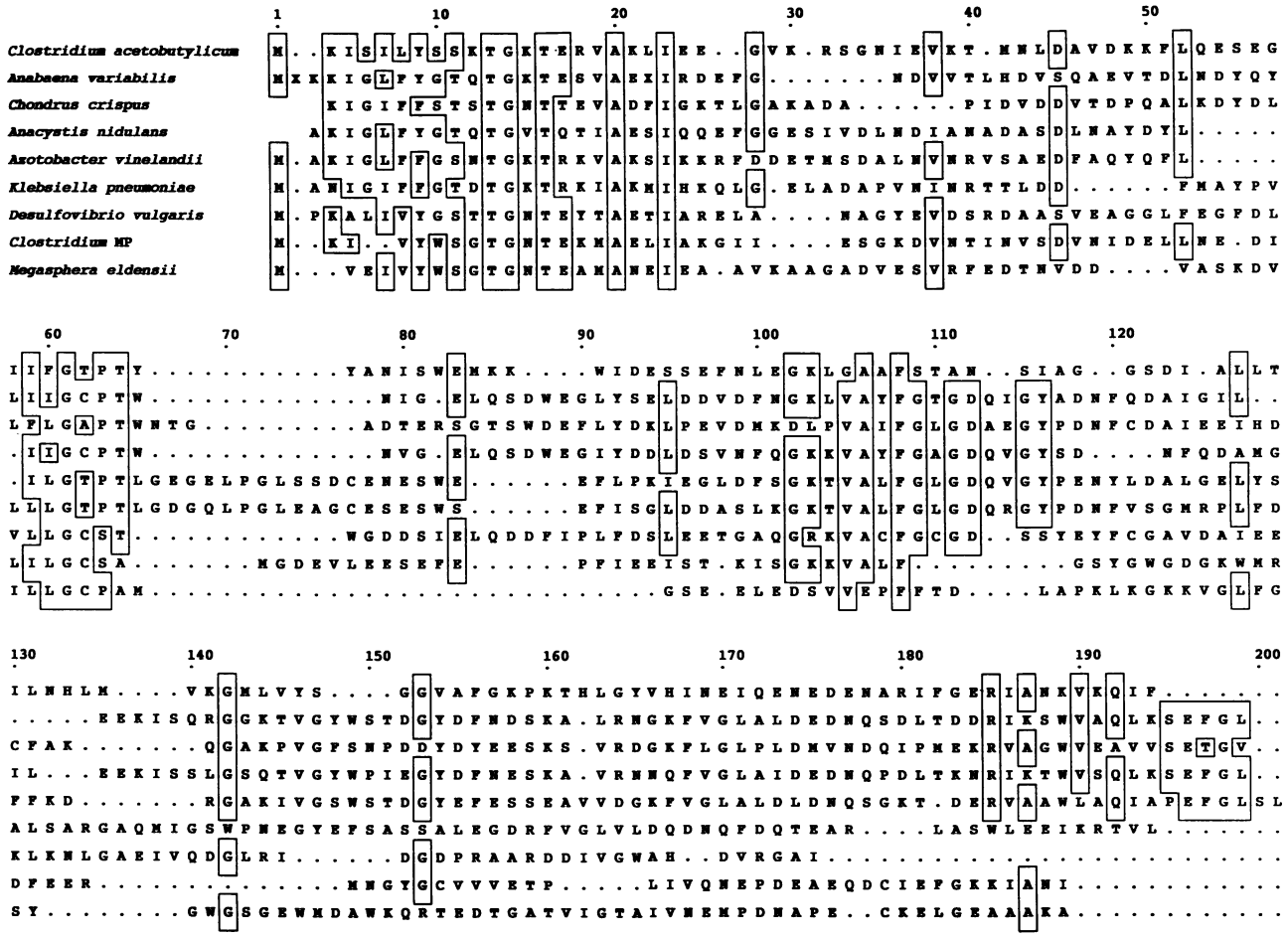

FIG. 5. Comparison of the amino acid sequence of the *C. acetobutylicum* flavodoxin with eight other flavodoxin amino acid sequences, from *Anabaena variabilis* (27), *Chondrus crispus* (46), *Anacystis nidulans* (25), *A. vinelandii* (2), *K. pneumoniae* (9), *D. vulgaris* (10, 24), *Clostridium* strain MP (41), and *Megasphera eldensii* (42). The boxed regions indicate sequences with identical amino acid residues. Chemically related amino acids are not indicated.

DISCUSSION

The *E. coli* F19 *recA*, nitrate reductase-deficient mutant proved to be a most suitable host for the isolation of recombinant plasmids, and screening experiments resulted in the isolation of 25 different recombinant plasmids. The *E. coli* F19 mutant can be utilized for the isolation of genes from other bacteria which are involved in the reduction of metronidazole. Furthermore, since we have isolated a *recA*$^+$ revertant of *E. coli* F19, we now have a battery of *E. coli* strains and *C. acetobutylicum* genes that can be used to test the activities of novel antimicrobial compounds related to metronidazole. It can be inferred whether a compound needs to be reduced to become active and whether the active compound is related to DNA repair by comparing the sensitivities of the various strains, with and without the cloned genes, to these novel compounds.

In choosing enzyme systems which may be controlled by the 25 plasmids, it was obvious that the activity of nitrate reductase should be analyzed. However, in strict anaerobes such as *C. acetobutylicum*, in which oxygen cannot be utilized as the terminal electron acceptor, other electron transport mechanisms also exist. Ferredoxin is a low-molecular-weight, iron-sulfur-containing protein which can accept and donate electrons at a very low potential approaching that of the hydrogen electrode (E_0 = −410 mV) (19). This molecule plays a crucial role as an electron carrier in electron distribution in the cell. Oxidized ferredoxin accepts electrons during the phosphoroclastic conversion of pyruvate to acetyl coenzyme A. Reduced ferredoxin can transfer electrons to the iron-containing hydrogenase, which uses protons as the final electron acceptor. Like ferredoxin, flavodoxin is a small acidic molecule that functions as a low-potential electron transfer protein (24). The redox potential of the semiquinone-hydroquinone couple is approximately −450 mV (31). Flavodoxin was first isolated from *C. pasteurianum* grown under iron-deficient conditions (20), and it was shown to be able to replace ferredoxin as an electron carrier (21, 22).

O'Brien and Morris (35) studied the effects of metronidazole on hydrogen production in *C. acetobutylicum*. These authors reported that when subinhibitory concentrations of metronidazole were added to *C. acetobutylicum* cultures, hydrogen evolution ceased until the presence of metronidazole could no longer be detected. Despite the inhibitory effect of metronidazole on hydrogen production, no alteration in carbon dioxide production occurred. This observation supports the theory of Church et al. (6) that hydrogenase

is the enzyme responsible for reducing metronidazole, the latter replacing protons as the final electron acceptor.

E. coli(pMET15B2) was the only transformant that demonstrated hydrogenase activity, which was approximately half of the activity of the wild-type *E. coli* Puig426 (Table 1). Voordouw et al. (44) reported very low levels of hydrogenase activity for the *Desulfovibrio vulgaris* hydrogenase cloned into *E. coli*. *E. coli* therefore may not be a suitable host for expressing hydrogenase activity and perhaps lacks the necessary electron carriers required by the *C. acetobutylicum* hydrogenase. The fact that *E. coli*(pMET15B2) was a class I isolate may be related to the low levels of hydrogenase activity. The fact that only 1 of the 25 different recombinant plasmids encoded an enzyme that was likely to be involved in metronidazole reduction suggests that either these enzyme activities are unstable and difficult to assay in *E. coli* or there are some interesting genes encoding proteins not previously considered to be involved in metronidazole reduction.

DNA hybridization and restriction endonuclease mapping revealed that pMET13A, pMET15B2, pMET030, and pMET190 were linked. The total length of chromosomal DNA from *C. acetobutylicum* represented by pMET13A, pMET15B2, pMET030, and pMET190 is approximately 11.1 kb. This 11.1-kb DNA fragment from *C. acetobutylicum* contained at least two genes involved with electron transfer systems capable of activating metronidazole. Further DNA sequencing of the cloned fragments and analysis of the gene products will indicate the presence or absence of other electron transfer genes. Regulatory studies will indicate whether the flavodoxin and hydrogenase genes are part of an electron transfer operon.

The insert DNAs in pMET190 and pMET13A are in opposite orientations with respect to the vector DNA, indicating that in *E. coli* this open reading frame is most likely transcribed from a promoter present on the insert. Although a putative promoter was identified between positions 124 and 160, this promoter does not have a high degree of identity to the gram-positive consensus sequence and other promoter sequences identified for *C. acetobutylicum* (17) and may not function in *C. acetobutylicum*. Further transcriptional studies are necessary to verify the true promoter region in both *E. coli* and *C. acetobutylicum*.

On the bases of the deduced amino acid sequence of this open reading frame and the ability of the gene product to activate metronidazole, we have identified the gene as a *C. acetobutylicum* flavodoxin gene. The amino acid sequences of eight other flavodoxins have been either determined by direct amino acid sequencing or deduced from their nucleotide sequences. Wakabayashi et al. (46) aligned seven of these flavodoxin sequences, and we used their alignment as a basis for the comparison shown in Fig. 5, which includes the *C. acetobutylicum* flavodoxin. The *C. acetobutylicum* flavodoxin shows some degree of similarity to the other flavodoxins. The invariant regions are located primarily at the flavin mononucleotide binding domains (the redox-active prosthetic group), which are included in positions 11 to 23, 58 to 64, and 101 to 114 (46). Glycine residues at positions 60, 142, and 152 may play a role in stabilizing the protein.

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