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ENVIRONMENTAL RESEARCH BRIEF

Genetic Engineering of Enhanced Microbial Nitrification

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Abstract

Experiments were conducted to introduce genetic information in the form of antibiotic or mercuric ion resistance genes into *Nitrobacter hamburgensis* strain X14. The resistance genes were either stable components of broad host range plasmids or transposable genes on plasmids presumably unable to replicate in strain X14. Four methods for plasmid transformation as well as conjugation with various donor strains of *Escherichia coli* failed to achieve this goal. We also undertook the cloning of an origin of replication from strain X14; seven such experiments were unproductive. Both the *leuB* and *thrB* gene. of strain X14 were successfully cloned by means of complementation of a *leuB thrB* auxotroph of *E. coli*. The *leuB* gene containing DNA was restriction-mapped and the 1.3 kilobase pair gene was subcloned into a vector suitable for use in DNA sequencing. To date, a tentative sequence comprising about 1300 bases has been obtained. There is extensive similarity in three regions of the sequence with the amino acid sequence of the *leuB* gene product of *Thermus thermophilus*, *Salmonella typhimurium*, and *Saccharomyces cerevisiae*.

Although the primary goal of developing a procedure for introducing genetic material into a nitrifying organism has not yet been achieved, the results achieved have produced useful information on the genomic organization of *Nitrobacter* as well as a plasmid-borne library of genes from that organism. Future experiments can be made with this library in order to provide additional basic information on *Nitrobacter's* genomic organization.

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Introduction

Nitrification, the conversion of ammonia to nitrite, is a desirable process in wastewater treatment facilities. Improvement of the nitrification process by genetic engineering of nitrifiers would provide a variety of benefits, e.g. nitrifiers needing less retention time, nitrifiers resistant to pollutants, nitrifiers active in cold weather, nitrifiers that grow more rapidly. The strains of nitrifiers required for such improvement could, in theory, be derived by genetic engineering.

Nitrification occurs in two steps: $NH_3 \rightarrow NO_2^- + H^+$ and $NO_2^- \rightarrow NO_3^-$. It is accomplished by the sequential action of two genera of autotrophic bacteria. Bacteria of the genera designated by the prefix "Nitroso" oxidize ammonia, liberated from organic matter by the action of heterotrophic bacteria, to nitrite. Nitrite is then oxidized to nitrate by autotrophs of the genera designated by the prefix "Nitro". The best known species are *Nitrosomonas europa* and *Nitrobacter winogradsky*. The term nitrifiers is used when referring to both genera collectively. Most publications focus primarily on the physiology, enzymology and structure-function relationships of the nitrifiers. Despite the important contribution of nitrifiers to wastewater treatment, as well as to the global nitrogen cycle, virtually nothing is known of their genomic organization.

Genetic engineering of bacteria usually involves either the introduction of foreign genes or the alteration of the existent bacterial genome. In either case, basic information on the organization and regulation of the bacterial genome is desirable. In this study, although such information on nitrifiers was lacking, it seemed reasonable to introduce, by conjugation or transformation, either an antibiotic or mercuric ion resistance plasmid into a nitrifier. This decision was based on the numerous successful introductions of resistance plasmids into Enterobacteriaceae and the ease of

positive selection for antibiotic and mercuric ion resistance. The stable antibiotic kanamycin and the stable mercuric ion were chosen as the selective agents since nitrifiers grow extremely slowly.

We choose to use *Nitrobacter hamburgensis* strain X14, henceforth simply strain X14 (4), in our studies because it grew relatively rapidly. Furthermore, it and the related strain Y (4), were the only *Nitrobacter* strains known to contain plasmids (14). Both strains contain three large (110, 186, and 273 kilobase) cryptic plasmids. This suggested that strain X14 would be physiologically able to maintain a newly introduced plasmid.

Procedures and Results

Introduction of Resistance Plasmids by Conjugation

The bacterial strains used in this study are listed in Table 1. *N. hamburgensis* strain X14 was known to be resistant to chloramphenicol (M. Pohl, unpublished observation). Therefore, a concentration of 50 µg/ml in agar solidified mixotrophic (21) or autotrophic (21) growth medium usually was used to counterselect against the *E. coli* plasmid donors. All matings between strain X14 recipients and *E. coli* donors were performed by mixing together the two organisms on a sterile 0.22µ filter at a donor to recipient ratio of 10 to 1. The filter was immediately placed on an agar plate of mixotrophic medium and incubated (30°C) for either 2,5,8, or 24 h to allow conjugation. The cells on the filter were harvested in either mixotrophic or autotrophic media, generally supplemented with chloramphenicol (50 µg/ml) and incubated for 24 h (30°C) to allow phenotypic expression of resistance. Cells were harvested by centrifugation, resuspended in a small volume, and the entire volume plated among several selective agar plates. The plates were placed in plastic sleeves, which were loosely sealed, and then incubated (30°C) for up to 30 days. Control platings of strain X14 formed minute colonies in ca 21 days. Despite precautions, fungal contamination occasionally occurred on some plates.

In only 1 of 13 instances, and then only in the form of a single colony, was the *E. coli* donor able to emerge on the selective plate.

The plasmids chosen for use (Table 2) were generally broad host range plasmids that carried an antibiotic resistance gene for a chemically stable antibiotic, e.g., kanamycin, streptomycin. The stability of the antibiotic was necessary because of the long incubation period. Other plasmids carried the transposon Tn 501 (mercuric ion resistance), which is useful since the mercuric ion in selective plates is chemically stable. Three of the plasmids were incapable of replicating except in *E. coli* and carried transposons that could transpose to either the chromosome or the endogenous plasmids of strain X14.

Although preliminary experiments with all donor stains showed them to be fertile when conjugated with *E. coli*, in no case were any resistant transconjugants of strain X14 isolated.

Introduction of Resistance Plasmids by Transformation

The introduction by transformation of two broad host range plasmids in strain X14 was attempted in two separate experiments. In each experiment, four different protocols were used (7,9,12,24). Three of the protocols (7,12,24) were selected since they had been used with recipient organisms resistant to the more traditional fourth procedure (9), which we also employed. After exposure to the resistance plasmid, the strain X14 cells were incubated in mixotrophic medium to allow phenotypic expression before plating on selective media. Although control experiments proved that the plasmids were capable of transforming *E. coli* with high efficiency, no resistant transconjugants of strain X14 were isolated.

Isolation of Origin of Replication

Strain X14 contains four origins of replication (*ori*): one chromosomal *ori* and an *ori* on each of the three cryptic plasmids. Our inability to introduce broad host range resistance plasmids into strain X14 may have been due to the incompatibility of their *ori* with the physiological properties of strain X14. We, therefore, designed the following experiments to clone an *ori* of strain X14. Chromosomal and plasmid DNA of strain X14 were digested separately to completion with the restriction endonucleases, *Bam*HI, *Pst*I, and *Sau*3A. The resultant fragments were ligated into digested vector pMK2004 to yield three gene libraries. Each library was used to transform *E. coli* JZ294, a *polA* strain. *PolA* strains are unable to use the ColEI-derived *ori* of the vector pMK2004 (Figure 1; ref. 13). They will, however, replicate vectors that contain a non-ColE1-derived ori (10,11). Transformation of *E. coli* JZ294 with the three gene libraries failed to yield any transformants. Because we used three different gene libraries and because previously identified *ori's* of Gram-negative organisms have all been relatively short sequences of ca 250 base pairs (27), our negative results suggest very strongly that the *ori* sequence(s) in *Nitrobacter* are non-functional in *E. coli*.

Construction of a Gene Library of Strain X14

The inability to introduce resistance plasmids into strain X14 and to isolate an origin of replication from strain X14 could be interpreted as follows. The regulatory sequences on the resistance plasmids that regulate transcription, translation, and DNA replication are those commonly used by Enterobacteriaceae. These regulatory signals may be distinct from those used by Nitrobacter for these physiological properties. To learn what regulatory sequences strain X14 uses to regulate transcription and translation, we undertook the isolation of the *leuB* gene from strain X14. Genomic DNA was prepared from strain X14 as follows. A suspension of 0.5 g cells in 6 ml TE (10 mM Tris, 1 mM EDTA; pH 8), 25 ml 2% sodium dodecyl sulfate, and 10 ml 0.35% proteinase K was incubated at 37°C for 2 h. The resultant viscious lysate was extracted twice with an equal volume of phenol and then twice with an equal volume of chloroform-isoamyl alcohol (24:1, v/v). The aqueous

Table 1. Bacterial Strains

Bacteria	Relevant bacterial genotype*	Source, reference or both
Nitrobacter hamburgensis		
X14	Prototrophic autotroph; 3 cryptic plasmids	E. Bock, Univ. of Hamburg, F.R.G.; (4)
X14-Sm^R	Spontaneous Sm^R mutant	This study
Nitrobacter agilis	Prototrophic autotroph	ATCC 14123
E. coli		
UW937 (pUW942)	*thr leu*	(25)
HB101 (pUW964)	*pro*	(26)
UB1636 (pMR5)	*his trp lys*	(19)
J53 (RP4)	*met pro*	J. C. Loper University of Cincinnati
MC1061 (pBR322 Tet::*Tn5*)	*leu*	J. Lodge Washington University St. Louis
C	*ilv*	J. C. Loper University of Cincinnati
K12 (RP4.8::*Tn*501)	Prototroph	M. Davidson University of Georgia
JZ294	*polA1*	D. Smith University of California at San Diego
C600	*leuB6 thrB thi*	(15)
CV438	leu*B61 thi pro*	J. M. Calvo Cornell University

*Relevant genotype of plasmids shown in Table 2.
Sm^R, streptomycin resistant.

phase was mixed with 0.6 volume isopropanol and kept at -20°C for 2 h. The precipitated DNA was recovered by centrifugation and dissolved in TE. After centrifugation in CsCl-ethidium bromide, the chromosomal DNA band was collected and dialyzed versus TE. The DNA was digested partially with *Sau*3A, and the resultant digestion mixture was electrophoresed in 1% low melting agarose. The 4 to 6 kilobase (kb) portion of the gel was cut out, and the DNA extracted. The DNA was ligated to *Bam*HI-digested vector pMK2004 (Figure 1) and the resultant ligation mixture was used to transform JZ279, a *rec*A derivative of LE 392 (15), by the method of Hanahan (9). We calculated that a total of 3990 kanamycin resistant transformants had been obtained; a unique copy of chromosomal DNA should be represented

Table 2. Plasmids Used or Derived in This Study

Plasmid	Relevant genotype*	Source or reference
pUW942	::*Tn*501 (Hg^R) Col E1 rep	(25)
pUW964	::*Tn*5 (Kan^R) Col E1 rep	(26)
pMR5	::*Tn*801 (Amp^R) rep^{ts}	(19)
RP4	Kan^R IncP	(11)
RP4.8::*Tn*501	Kan^R IncP	Michael Davidson University of Georgia
pBR322Tet::*Tn5*	::*Tn*5 (Kan^R) Col E1 rep	Jennifer Lodge Washington University
pCV57	$leuA^+B^+C^+D^+$	J. M. Calvo Cornell University
pNBH1,6	$leuB^+$ Kan^R	This study
pNBH3,8,10,11	$leuB^+$ Kan^R	This study
pNBH601	$leuB^+$ Kan^R	This study
pNBH602	$leuB^+$ Kan^R	This study

* Genetic symbols: Hg^R, mercuric ion resistant; Kan^R, kanamycin resistant; Amp^R, ampicillin resistant; rep^{ts}, replication temperature sensitive; IncP, incompatibility group P; Col E1 rep, Colicin E1 replication.

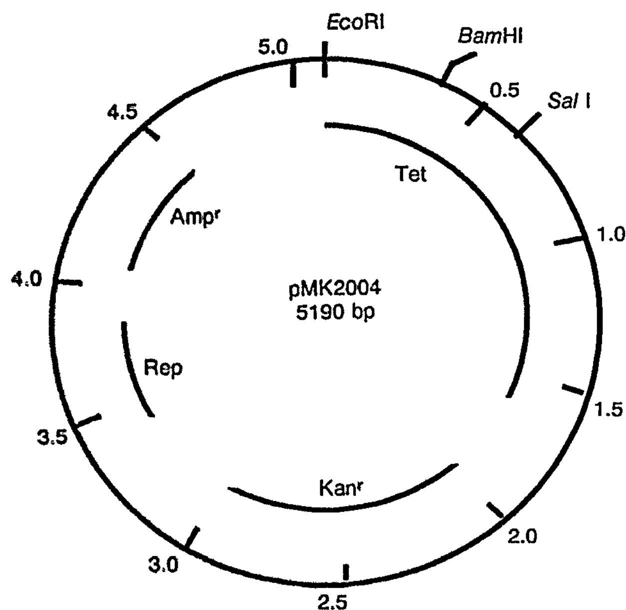

Figure 1. Map of relevant restriction sites in pMK2004. Modified from reference 13. Coordinates are in kilobase pairs. Tet, tetracycline resistance gene; Kan^r, kanamycin resistance gene; Amp^r, ampicillin resistance gene; Rep, origin of replication.

at least once in the library with 99% confidence (5). This conclusion was based on the assumption that the chromosome of strain X14 and that of *E. coli* are of equal size. Recombinant plasmid DNA, prepared from the transformed cells by the method of Birnboim and Doly (3), was purified by CsCl-ethidium bromide gradient centrifugation.

Complementation of E. coli *C600*

Recombinant plasmid DNA was mixed with competent (9) cells of *E. coli* C600, a *leuB6 thrB* mutant. Part of the mixture was plated on leucine-deficient and part on threonine-deficient agar medium. Twelve Leu$^+$ transformants and three Thr$^+$ transformants were so isolated. Six of the *leuB6* and one of the *thrB* complementing plasmids were stable; the reason for the instability of the other plasmids is unknown. No further work was done with the *thrB*-complementing plasmid.

Southern Analysis

It was necessary to prove that the complementing activity in the recombinant plasmids was due to *Nitrobacter* DNA rather than contaminating *E. coli* DNA present in the pMK2004 preparation. *Nitrobacter* genomic DNA and *E. coli* C600 DNA were digested completely with *Eco*RI and *Sal*I, resolved on agarose gels, transferred to nitrocellulose, and probed with nick translated pNBH6 and pMK2004. The radiolabeled pNBH6 did not hybridize to *E. coli* DNA but did hybridize to a *Nitrobacter* DNA restriction fragment of the appropriate size, and to the vector pMK2004. As a necessary negative control, we also showed that the vector pMK2004 failed to hybridize to *Nitrobacter* DNA. This Southern analysis confirmed our conclusion that the complementing activity of pNBH6 was due to *Nitrobacter* DNA.

Plasmid Coded Enzymatic Activity

The *leuB* gene of *E. coli* codes for β-isopropylmalate dehydrogenase (6,20). This enzymatic activity should, therefore, be present in soluble extracts made from *E. coli* C600 strains which contain a *leuB*-complementing plasmid. As seen in Table 3, enzymatic activity was present in recombinant plasmid bearing strains but absent in two *E. coli leuB* strains. Since *E. coli* CV438 (*leuB*61) and C600 (*leuB*6) contain different *leuB* mutant alleles (18), the expression of enzymatic activity when they bear the same complementing plasmid argues against *leuB* complementation by *Nitrobacter* DNA being due to a *Nitrobacter* suppressor tRNA.

Table 3. Β-Isopropylmalate Dehydrogenase Activity of *E. coli* strains

Expt. No.	Strain (plasmid)	Leucine in growth medium (µg/ml)*	Specific activity$^+$
1	CV438 (pMK2004)	40	<0.001
	CV438 (pCV57)	0	0.057
	CV438 (pNBH1)	0	0.040
	CV438 (pNBH3)	0	0.017
	CV438 (pNBH6)	0	0.053
2	K12	0	0.084
	C600 (pMK2004)	10	<0.001
	C600 (pMK2004)	40	<0.001
	C600 (pNBH6)	0	0.10
	C600 (pNBH601)	0	0.162
	C600 (pNBH602)	0	0.137

* Minimal salts medium (23) was supplemented with proline and thiamine (Expt. 1) or with threonine and thiamine (Expt. 2).
$^+$ Micromoles α-ketoisocaproate formed min^{-1} mg protein^{-1}.

To obtain additional evidence that the leucine-complementing activity was not due to inadvertent cloning of *E. coli* DNA, the soluble extracts were electrophoresed by continuous polyacrylamide gel electrophoresis under nondenaturing conditions. β-isopropylmalate dehydrogenase activity was detected by a histochemical stain (22) in extracts prepared from *E. coli* strains that bore complementing plasmids but not in those prepared from *E. coli* C600 (pMK2004). The relative mobility of the cloned *Nitrobacter* β-isopropylmalate dehydrogenase was slower than that of *E. coli.*

Complementation of leuA, C, *and* D *Mutants*

The four leucine biosynthetic genes occur as a cluster of four contiguous genes, i.e., as an operon, in both *Salmonella typhimurium* (16) and *E. coli* (20). The amount of DNA needed to code for the four *S. typhimurium leu* polypeptides was estimated to be ca 4.1 kb (6).

Our DNA sequencing data (see below) indicated that the *leuB* gene existed within the 1.4 kb *Bam*HI-*Sma*I fragment of pNBH602 (Figure 2). Thus, there was sufficient DNA on

either side of the comparable region in pNBH3 (Figure 2) for one or more of the other three *leu* genes. We, therefore, transformed a *leuA*, a *leuC*, and a *leuD* mutant of *E. coli* with pNBH3. Plating on kanamycin-supplemented agar indicated that all three of the mutants were successfully transformed by pNBH3. None of the three mutants had, however, been transformed to leucine independence. We concluded that the four leucine genes in *Nitrobacter* are not organized as an operon.

Restriction Mapping and Subcloning

The size of the insert in the six stable *leuB*-complementing plasmids was either ca 12.5 kb (pNBH1, pNBH6) or ca 6.7 kb (pNBH3, pNBH8, pNBH10, pNBH11). We identified a common 3.7 kb *Bam*HI-*Eco*RI restriction fragment that perforce contained the *leuB*-complementing activity (Figure 2). This fragment was isolated from a *Bam*HI, *Eco*RI digest of pNBH6 and ligated into similarly digested pMK2004. The resultant plasmid, pNBH601 (Figure 2), complemented the *leuB* mutation in *E. coli* C600. The reverse orientation of the 3.7 kb *Bam*HI-*Eco*RI fragment in pNBH6 and pNBH601 (footnote Figure 2) suggested strongly that transcription originated at a promoter site within the cloned *Nitrobacter* DNA. Further subcloning by means of *Bam*HI-*Sal*I digestion of pNBH6 and subsequent ligation into pMK2004 yielded the *leuB*-complementing plasmid pNBH602 (Figure 2). The resultant 2.4 kb *Bam*HI-*Sal*I fragment of pNBH602 was isolated and a restriction map constructed (Figure 2).

Sequencing the leuB Gene of Strain X14

The *leuB* gene had been localized within the 2.4 kb *Bam*HI-*Sal*I fragment of pNBH602 (Figure 2). We subcloned the ca 1.4 kb *Bam*HI-*Sma*I portion of that fragment in both orientations into pAA 3.7X (1). The method described by Ahmed (1) was used to generate sets of overlapping deletions suitable for sequencing both strands of the DNA fragment. To date, a total of 1311 bases have been sequenced from both strands. At this point, two aspects of the derived amino acid sequence are especially noteworthy. First is the occurrence of three regions of homology among the amino acid sequences of the β-isopropylmalate dehydrogenase of strain X14, *S. typhimurium* (J. Calvo, pers. comm.), *Thermus thermophilus* (17), and *Saccharomyces cerevisiae* (2) (Figure 3). Second is that the cited correspondence between the strain X14 amino acid sequence and the other amino acid sequences allows us to predict that the *Bam*HI-*Sma*I fragment contains approximately 120 bases of 5'-flanking and approximately 150 bases of 3'-flanking DNA. This amount of flanking DNA should presumably encompass the sought for 5'-flanking (promoter and ribosome-binding) and 3'-flanking (transcription termination) sequences (8).

Conclusions

The original goal of introducing genetic information into strain X14 has not been realized. One plausible interpretation of the inability to transfer genetic information by either transformation or conjugation is that the replication and/or transcriptional and/or translational signals in the various plasmids are not functional in strain X14. A second possibility is that foreign DNA can not enter by the biological transfer mechanisms of conjugation and transformation. Efforts to clone the four replication origins

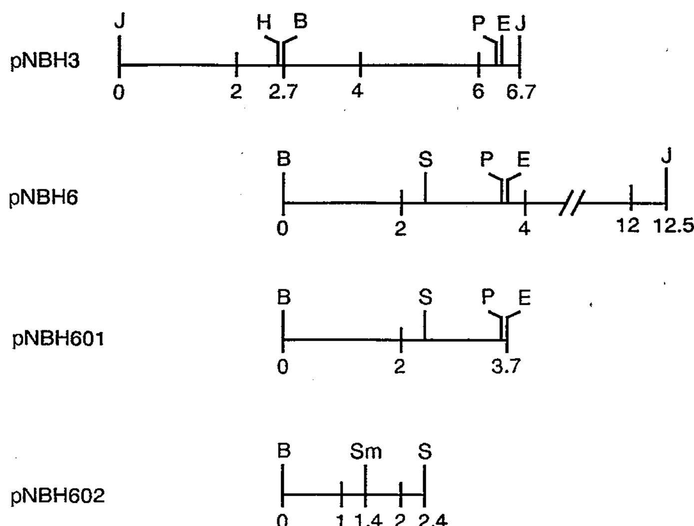

Figure 2. Relevant restriction sites in *leuB*-complementing fragments derived from genomic DNA of strain X14 or by subsequent subcloning. Each of the fragments when cloned into appropriately digested vector pMK2004 was shown to be *leuB*-complementing. The coordinates are in kilobase (kb) pairs and are approximate values. The *leuB*-complementing fragments of pNBH1 and pNBH6 are assumed to be identical, as are those of pNBH3, pNBH8, pNBH10 and pNBH11. Note that the 3.7 kb *Bam*HI-*Eco*RI fragment in pNBH6 and pNBH601 are in opposite orientations within their respective plasmids. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; J, *Bam*HI/*Sau*3A; P, *Pst*I; S, *Sal*I; Sm, *Sma*I.

found in strain X14 were unsuccessful. The successful cloning of the *leuB* gene of the strain X14, the first gene cloned from a nitrifier, provided us with the opportunity to examine the flanking regions of this gene for the presence or absence of traditional transcriptional and translational signals common in the enterobacteria. To date, a total of ca 1300 bases of the *leuB* gene of strain X14 have been sequenced. Since these data are a composite of sequences from both strands, contain ambiguous bases, and do not yet include the two junction fragments where the subcloned gene joins the vector, no definitive statement can be made at this time about the sought-for transcriptional and translational DNA sequences. However, three sequences of amino acids derived from the DNA sequence contain, respectively, 13, 9, and 18 amino acids that are either identical or functionally equivalent to sequences reported in the same enzyme from *T. thermophilus, S. typhimurium*, and *S. cerevisiae.* No function(s) have been ascribed to any of the amino acid sequences in the enzymes from the latter three enzymes.

Recommendations

This research has provided the first basic information on the genomic organization of a nitrifying organism, *N. hamburgensis* strain X14. It is apparent that the *leuB* biosynthetic enzyme of *Nitrobacter* shares considerable

a (9) AlaValLeuPheGlyAlaValGlyGlyProLysTrpAsp (21)
b (64) AlaValLeuLeuGlySerValGlyGlyProLysTrpAsp (76)
c (68) AlaIleLeuPheGlySerValGlyGlyProLysMet (79)
d (71) AlaValLeuLeuGlyAlaValGlyGlyProLysTrp (82)

a (40) LeuTyrAlaAsnLeuArgProAla (47)
b (97) LeuPheAlaAsnLeuArgProAla (104)
c (102) LeuPheSerAsnLeuArgProAla (109)
d (101) LeuTyrAlaAsnLeuArgPro (108)

a (67) ValAspIleMetIleValArgGluLeuThrXxxGlyValTyrPheGlyGluProLys (85)
b (124) ValAspValLeuIleValArgGluLeuThrGlyGlyIleTyrPheGlyGluProArg (142)
c (130) PheAspIleLeuCysValArgGluLeuThrGlyGlyIleTyrPheGlyGlnProLys (148)
d (130) ThrAspPheValValValArgGluLeuValGlyGlyIleTyrPheGly (145)

Figure 3. Comparison of derived amino acid sequences of the *leuB* DNA sequences of (a) strain X14, (b) *T. thermophilus,* (c) *S. typhimurium*, and (d) *S. cerevisiae*. Xxx, ambiguous sequence; numbers in parenthesis indicate amino acid residue.

amino acid homology with the same enzyme in two other Gram-negative bacteria and a yeast.

The partially completed sequencing of the *leuB* gene and its flanking regions should be completed. The resultant data will establish whether the DNA sequences which serve as signals in transcription and translation of *Nitrobacter* DNA, are similar to those found in other bacteria. Should the completed analysis of the DNA flanking the *leuB* gene fail to reveal any regulatory sequences common to other bacteria, then other *Nitrobacter* genes should be isolated and sequenced. The gene library of strain X14 constructed during this study is available as a source of other *Nitrobacter* genes. A comparison of the flanking DNA sequences would reveal sequences common to *Nitrobacter* genes; these would be prime candidates for the sought for regulatory sequences. This information is critical for the following reason. The inability to introduce resistance plasmids into strain X14 may be because incompatible regulatory sequences prevent expression of the resistance gene. The identification of *Nitrobacter*-specific regulatory sequences would allow the modification of plasmid vectors so that expression of an introduced resistance gene would be assured.

Although resistance plasmids could not be introduced into strain X14 by transformation or conjugation, their introduction by electroporation should be attempted.

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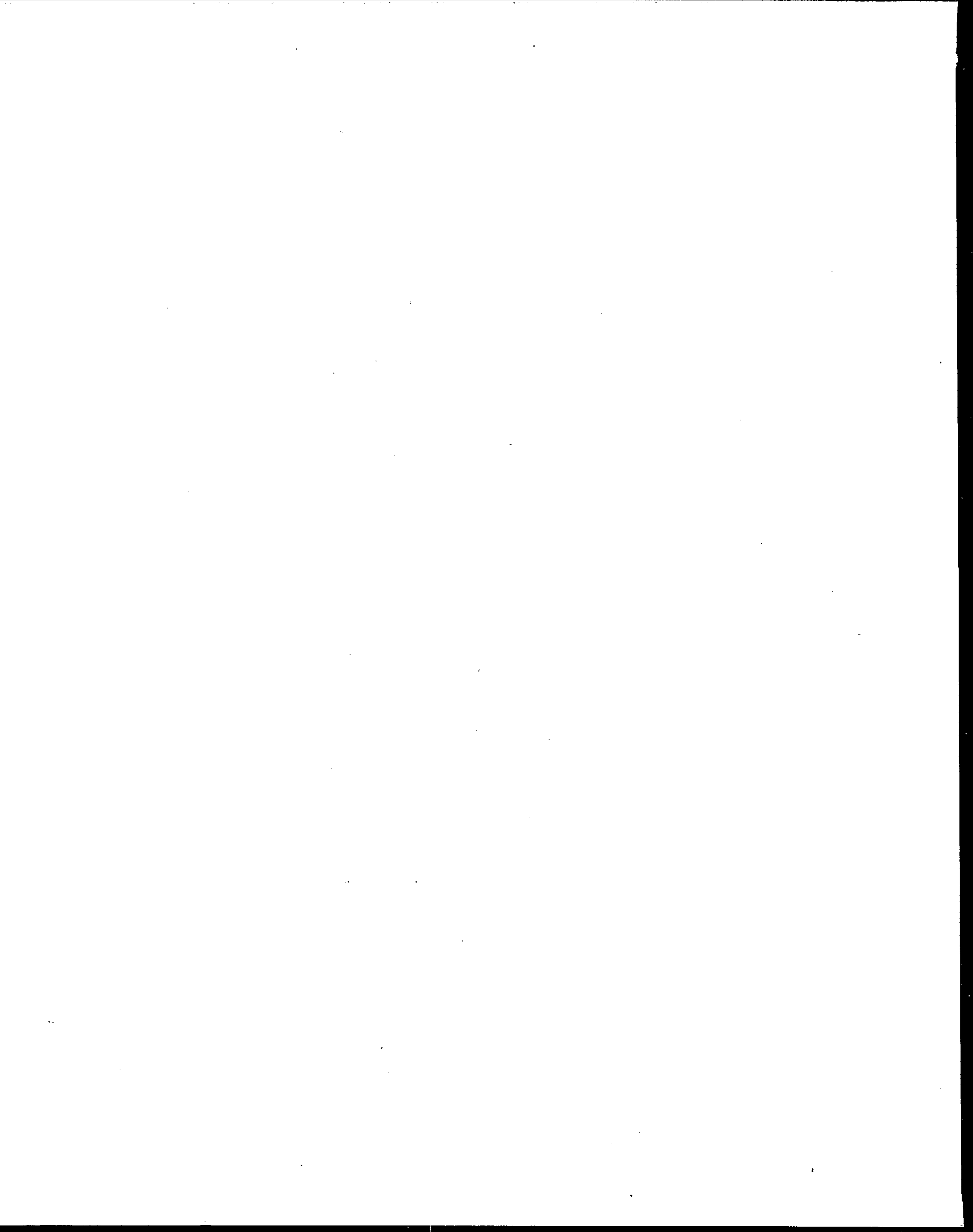

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PERMIT No. G-35

Official Business
Penalty for Private Use $300

EPA/600/M-89/011