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Cloning and Characterization of the Genes Encoding Nitrilotriacetate Monooxygenase of *Chelatobacter heintzii* ATCC 29600

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A 6.2-kb DNA fragment containing the genes for the nitrilotriacetate (NTA) monooxygenase of *Chelatobacter heintzii* ATCC 29600 was cloned and characterized by DNA sequencing and expression studies. The nucleotide sequence contained three major open reading frames (ORFs). Two of the ORFs, which were oriented divergently with an intergenic region of 307 bp, could be assigned to the NTA monooxygenase components A and B. The predicted N-terminal amino acid sequences of these ORFs were identical with those determined for the purified components. We therefore named these genes *ntaA* (for component A of NTA monooxygenase) and *ntaB* (for component B). The *ntaA* and *ntaB* genes could be expressed in *Escherichia coli* DH5α, and the gene products were visualized after Western blotting (immunoblotting) and incubation with polyclonal antibodies against component A or B. By mixing overproduced NtaB from *E. coli* and purified component A from *C. heintzii* ATCC 29600, reconstitution of a functional NTA monooxygenase complex was possible. The deduced gene product of *ntaA* showed only significant homology to SoxA (involved in dibenzothiophene degradation) and to SnaA (involved in pristamycin synthesis); that of *ntaB* shared weak homologies in one domain with other NADH:flavine mononucleotide oxidoreductases. These homologies provide no conclusive answer as to the possible evolutionary origin of the NTA monooxygenase. The deduced gene product of the third ORF (ORF1) had homology in the N-terminal region with the GntR class of bacterial regulator proteins and therefore may encode a regulator protein, possibly involved in regulation of *ntaA* and *ntaB* expression.

The extensive use of the synthetic chelator nitrilotriacetate (NTA) in different domestic and industrial applications has led to considerable concern about the potential of NTA to remobilize heavy metals from sediments and activated sludge, which could result in a contamination of drinking water with metal-NTA complexes (1). Fortunately, NTA appears to be relatively easily degradable, and the concentrations of NTA in surface waters remain low despite an increased use. NTA degradation was shown to be a result of microbial processes rather than of photochemical degradation (1, 3, 6, 17, 29, 33). Several microorganisms, either obligately aerobic or facultatively denitrifying, that can use NTA as a sole source of carbon, nitrogen, and energy have been isolated (3, 10, 19, 23, 30, 46, 53). The majority of the obligately aerobic isolates has been assigned to two new genera, *Chelatobacter* and *Chelatococcus* (2).

The biochemical pathway for NTA degradation was first investigated in the two virtually identical "chelatobacter" isolates T23 (10, 24) and ATCC 29600 (22). In both strains, a monooxygenase was responsible for the oxidative cleavage of NTA to iminodiacetate (IDA) and glyoxylate. This monooxygenase was proposed to catalyze the hydroxylation of an α-carbon atom of NTA, which then spontaneously forms IDA and glyoxylate (Fig. 1) (22). Initial attempts to purify this NTA monooxygenase resulted in the complete loss of NTA cleavage activity although the isolated protein fraction showed NTA-stimulated NADH oxidation (21, 44). Recently, the functionally active NTA monooxygenase complex from *Chelatobacter heintzii* ATCC 29600 was isolated and characterized (51). The purified NTA monooxygenase consisted of two components, A and B, which occurred as a tetramer ($\alpha_2\beta_2$). On sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), the molecular masses of the monomers of components A and B (cA and cB) were 47 and 36 kDa, respectively. cB is a flavin mononucleotide (FMN)-containing protein with NTA- and Mg^{2+}-stimulated NADH-oxidizing activity. The role of cA remained unclear, but its presence was absolutely necessary for the α-hydroxylation of NTA and the formation of IDA, glyoxylate, and H_2O. Interestingly, no compounds other than NTA have been found to serve as substrates for the NTA monooxygenase. The enzyme may be distributed in other *Chelatobacter* and *Chelatococcus* strains as well, since proteins reacting specifically with antibodies raised against the two-component NTA monooxygenase could be detected in most of the *Chelatobacter* and *Chelatococcus* strains tested (49). However, at least in one NTA degrader, *Chelatococcus asaccharovorans* TE2, a protein cross-reacting with anti-cA antibodies, but none reacting with anti-cB antibodies, was present (49).

In this report, we describe the cloning and characterization of the genes coding for the NTA monooxygenase of *C. heintzii* ATCC 29600. The genetic organization of the two genes for NTA monooxygenase appears to be unusual, and their primary sequences indicate an unknown evolutionary origin and a unique enzyme system.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *C. heintzii* ATCC 29600 was grown on synthetic medium containing 1 g of NTA per liter as the only source of carbon, nitrogen, and energy as described earlier (19). *Escherichia coli* cells were cultivated at 37°C in Luria broth (LB) (40). Where required, the following compounds were added to the media: ampicillin (50 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; 30 μg/ml), and isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM).

N-terminal amino acid sequencing. Previously purified cB (0.9 nmol) of the NTA monooxygenase from *C. heintzii* ATCC 29600 (51) was bound to a polyvinylidene difluoride membrane by centrifugation at 5,200 × *g* to dryness in a Pro Spin Preparation cartridge (Applied Biosystems, Foster City, Calif.). Amino acid

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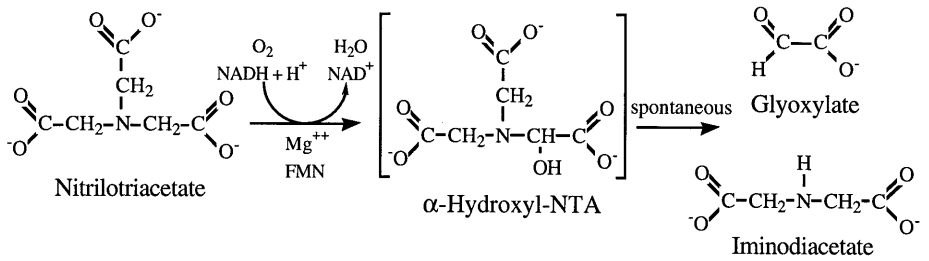

FIG. 1. Proposed reaction catalyzed by the two-component NTA monooxygenase in *C. heintzii* ATCC 29600.

sequencing was performed by automated Edman degradation on a model 475A peptide sequencer (Applied Biosystems). Phenylthiohydantoin amino acids were identified with a 120A on-line amino acid analyzer (Applied Biosystems). The N-terminal sequence of cA had been determined previously (51).

DNA amplification by PCR. The PCR was performed in a reaction mixture of 50-μl total volume containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.05% W1 (Life Technologies Inc., Gaithersburg, Md.), 0.2 mM each dATP, dCTP, dGTP, and dTTP, 1 ng of each primer DNA, 20 ng of template DNA, and 0.8 U of *Taq* DNA polymerase (Life Technologies). An amplification program of 30 cycles was run on a Crocodile II thermocycler (Appligene, Illkirch, France) with an annealing temperature of 42°C for 30 s, an extension temperature of 72°C for 1.5 min, and a denaturation temperature of 93.5°C for 1 min. The primers used in the PCR were 921003 (5′-GGIGCIGAIAAICAIATGAAIITIG G-3′) for the N terminus of cA of NTA monooxygenase and 890912 (5′-TGCA GCCATGTTCCCTG-3′) for the linker DNA.

DNA-DNA hybridizations. Hybridizations with total genomic DNA were performed on membrane-bound DNA, blotted from agarose gels to a QIABRANE membrane in a vacuum transfer apparatus (Pharmacia LKB Biotechnology, Uppsala, Sweden) at 50 mbar. Transfer was carried out by immersing the gel in (i) 0.25 M HCl for 10 min, (ii) a solution of 0.4 M NaOH and 0.6 M NaCl for 10 min, (iii) a solution of 1 M Tris-HCl (pH 7.6) and 1.5 M NaCl for 10 min, (iv) 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (37) for 30 min. After completion of the transfer, the membrane was rinsed with a solution of 0.4 M NaOH and 0.6 M NaCl for 30 s and then washed with a solution of 1 M Tris-HCl (pH 7.6) and 1.5 M NaCl for 1 min. The membrane was then air dried, illuminated with UV at 315 nm for 2 min, and subsequently prehybridized in hybridization buffer containing 7% SDS, 1% bovine serum albumin (BSA; fraction V), 0.5 M sodium phosphate (pH 7.2), and 1 mM EDTA (pH 8) for 1 h at 62°C. Hybridization was carried out at 42°C for 12 h with a radioactive labeled probe. As a probe, we used oligonucleotide 921003, which was 5′ labeled with [γ-^{32}P]dATP (3,000 Ci/mmol; Amersham, Little Chalfont, England) by using T4 polynucleotide kinase. Colony blotting of recombinant colonies was performed on Colonyscreen membranes (Dupont, NEN Research Products, Boston, Mass.) as instructed by the manufacturer. Hybridizations were then carried out as described above except that a different probe (a random-primed labeled 500-bp PCR fragment) and a different hybridization temperature (62°C) were used.

DNA manipulations and sequence analysis. Standard molecular cloning procedures were followed for genomic or plasmid DNA isolations, for transformations, and for enzymatic DNA manipulations (40). Restriction endonucleases and other DNA-modifying enzymes were purchased from Appligene, Life Technologies, or Pharmacia LKB Biotechnology. DNA fragments to be sequenced were cloned into M13mp18 or M13mp19 (54) and propagated in *E. coli* TG1 (40). The sequencing strategy is shown in Fig. 2. DNA sequencing was performed by the dideoxy-chain termination method of Sanger et al. (41) on a model 4000L automated DNA sequencer (LI-COR, Lincoln, Neb.). Cycle sequencing reactions were carried out with a SequiTherm Cycle sequencing kit (Epicentre Technologies, Madison, Wis.) with infrared fluorescently labeled universal primers (IRD41; LI-COR) as recommended by the manufacturer. Computer analysis and processing of sequence information were done with the programs PC/GENE (Genofit, Geneva, Switzerland) and GCG (University of Wisconsin) (13).

Western blot (immunoblot) analysis. Expression of the *nta* genes was tested in *E. coli* DH5α. For this, the strains with the different plasmid constructs (Fig. 2) were grown until an optical density at 546 nm of approximately 1 was reached. Samples of 1 ml were withdrawn from the culture suspension, and cells were harvested by centrifugation at 13,000 rpm for 10 min. Pellets were resuspended in 100 μl of Laemmli sample buffer. After boiling for 3 min, the protein samples were run on an SDS–12.5% polyacrylamide gel and blotted onto nitrocellulose as described elsewhere (40). Blocking, antibody incubations, and detection were then performed according to the ECL immunodetection protocol (Amersham). As primary antibodies, polyclonal antibodies raised against purified cA or cB were used (49). The polyclonal antibodies were purified as described by Harlow and Lane (26).

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant properties	Reference or source
Strains		
C. heintzii ATCC 29600	NTA^+	19
E. coli		
BL21(DE3)	F^- *hsdS gal* r_B^- m_B^- (*lacUV5*-T7 gene 1)	45
DH5α	F^- *lacZ* ΔM15 *recA1 hsdR17 supE44* Δ(*lacZYA-argF*)	Life Technologies
TG1	F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ* ΔM15] *supE hsd*Δ5 Δ(*lac-proAB*)	40
Plasmids		
pUC18/pUC19	Ap^r	54
pET8c	Ap^r	39
pNTA1	2.8-kb *Sac*I-*Bam*HI of pNTA9 in pUC18, Ap^r	This work
pNTA3	3.7-kb *Hin*dIII fragment of *C. heintzii* in pUC19, containing *ntaA*, ORF1, and part of *ntaB* (isolated by colony hybridization)	This work
pNTA4	2.4-kb *Pst*I-*Hin*dIII fragment of pNTA3 in pUC19	This work
pNTA5	1.9-kb *Xho*I fragment of pNTA3 in pUC19	This work
pNTA6	3.2-kb *Hin*dIII-*Sal*I fragment of pNTA3 in pUC19	This work
pNTA7	Same as pNTA6, opposite orientation of insert	This work
pNTA8	Same as pNTA3, opposite orientation of insert	This work
pNTA9	4.5-kb *Sac*I fragment of *C. heintzii* in pUC19, containing *ntaB* and part of *ntaA* (isolated by colony hybridization)	This work
pNTA10	Same as pNTA5, opposite orientation of insert	This work
pNTA17	1.9-kb *Nde*I (filled in)-*Bam*HI fragment of pNTA1 in pET8c, containing *ntaB* under control of T7 promoter	This work
pNTA21	2.0-kb *Nco*I-*Bgl*II fragment of pNTA3 in pET8c, containing *ntaA*; *Nco*I site introduced at the start site of *ntaA* by PCR	This work

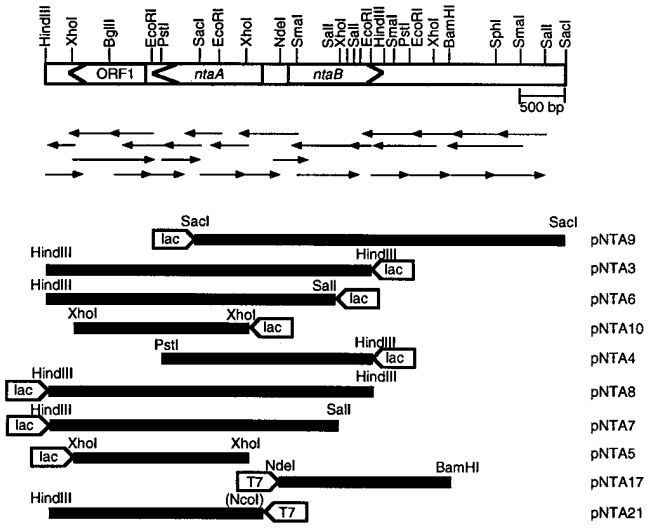

FIG. 2. Physical map of the analyzed region of *C. heintzii* and plasmid constructs used in this study. The upper part shows the isolated 6.2-kb DNA fragment and the locations of *ntaA*, *ntaB*, and ORF1. The small arrows below indicate the directions and sizes of the determined sequences. The fragment inserts of the different plasmids used for overexpression are shown in the lower part. The direction of transcription from the vector-located promoters (P_{lac} and T7) is shown by an open arrow. Relevant restriction sites are depicted.

Preparation of cell extracts. Cell extracts of *C. heintzii* ATCC 29600 to be tested for NTA monooxygenase activity were prepared from 200-ml cultures grown on NTA to late exponential phase. Cells were collected by centrifugation, washed twice with 30 mM Tris-HCl buffer (pH 8), and resuspended in 500 μl of a lysis buffer containing 30 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM DNase I. Cells were then disrupted by sonification four times for 1 min each on ice with a 30-s break between each cycle (Branson Sonifier 450, duty cycle 30%, output 3). Suspensions were subsequently centrifuged at 20,000 × *g* for 15 min at 4°C. The supernatant was collected and is referred to as the cell extract. Similarly, cell extracts of *E. coli* BL21(DE3) with plasmid pET8c, pNTA17, or pNTA21 were made from 150-ml cultures. These cultures were grown on LB with ampicillin and induced at an optical density at 546 nm of 0.3 by adding IPTG. After 4 h, the cells were harvested and cell extracts were prepared.

Determination of NTA monooxygenase activity. NTA monooxygenase in cell extracts was assayed by the method of Uetz et al. (51) except that we used an incubation buffer containing 30 mM Tris-HCl (pH 8), 10 mM FMN, and 2 mM $MgCl_2$. The NTA monooxygenase reaction was stopped by the addition of 0.4 volume of 25 mM HCl. The concentration of NTA in the assay was measured by high-pressure ion exclusion chromatography as described previously (42). Glyoxylate produced in the enzyme reaction was measured with the phenylhydrazine-$K_3Fe(CN)_6$ method (48).

Protein determination. Concentrations of protein in cell extracts were determined as described by Bradford (8), with BSA as the standard.

Nucleotide sequence accession number. The nucleotide sequence of the NTA monooxygenase genes determined in this study has been deposited in the GenBank database under accession number U39411.

RESULTS

Cloning of the NTA monooxygenase genes. To clone the genes for NTA monooxygenase, we performed hybridizations with an oligonucleotide which was designed according to the N-terminal amino acid sequence of purified cA (Table 2) (51). First, genomic DNA was isolated from NTA-grown cells of *C. heintzii* ATCC 29600 and digested with different restriction endonucleases. DNA fragments with the gene for the NTA monooxygenase cA were then identified by DNA-DNA hybridization with the degenerated oligonucleotide 921003. A 1.5-kb *Eco*RI, a 4-kb *Hin*dIII, and a 4.5-kb *Sac*I fragment hybridized to the 921003 probe. To obtain a more specific probe for the hybridizations, we decided to use PCR amplification. To this end, we isolated *Eco*RI fragments of about 1.5 kb, which hybridized with the 921003 probe, from the genomic DNA. These fragments were then ligated with a known 50-bp DNA fragment, which functioned as a linker. The ligation mixture was then subjected to PCR with one primer for the N-terminal sequence of cA (921003) and one primer for the linker sequence (890912). With this procedure, a DNA fragment of 500 bp was amplified, sequenced, and found to contain the start of the gene for cA of the NTA monooxygenase. This 500-bp DNA fragment was then used to screen a genomic minilibrary containing *Hin*dIII fragments of sizes between 3.5 and 4.5 kb in pUC19 and another containing 4- to 5-kb *Sac*I fragments of *C. heintzii* ATCC 29600. Several positive colonies could be detected by hybridization, and the plasmids contained in these clones were subsequently isolated and verified by DNA-DNA hybridization. In this manner, we cloned two overlapping DNA fragments of the genome of *C. heintzii* ATCC 29600 (Fig. 2), spanning 6.2 kb of DNA and containing the genes for NTA monooxygenase.

Nucleotide sequence analysis of the NTA monooxygenase genes. The isolated 6.2-kb DNA fragment was further characterized by restriction site mapping and subsequent DNA sequencing of suitable DNA fragments cloned into M13mp18 (Fig. 2). The complete nucleotide sequence of a 5,932-bp *Hin*dIII-*Sal*I DNA region was determined on both strands (Fig. 3). The nucleotide sequence revealed the presence of three major open reading frames (ORFs). On the basis of the N-terminal amino acid sequence of the purified proteins (Table 2), two of the ORFs could be assigned to the NTA monooxygenase components A and B, and the genes were named *ntaA* and *ntaB*, respectively. The two genes were divergently transcribed, with an intergenic region of 307 bp. The *ntaA* gene, located on the reverse strand between nucleotides 1123 and 2484, codes for a protein of 453 amino acids with a calculated mass of 50.5 kDa and a calculated isoelectric point of 6.5. The calculated size agrees well with the apparent size of 47 kDa observed with SDS-PAGE in a previous work of our group (51). The start codon at position 2484 is preceded by a putative ribosome-binding site GGAG at position 2497. The *ntaB* gene (positions 2792 to 3760) encodes a protein of 322 amino acids with a calculated mass of 34.5 kDa and a calculated isoelectric point of 5.5. This agrees very well with the apparent size of 36 kDa determined for NtaB with SDS-PAGE (51). Also, the start codon at position 2792 is preceded by a putative ribosome-binding site GGAG at position 2778. Between bases 282 and 914, a third, larger ORF, designated ORF1, was found. At present, no conclusive start codon can be determined for ORF1. Either an ATG at position 914 or a

TABLE 2. Comparison of the N-terminal amino acid sequences of purified cA and cB of NTA monooxygenase with those encoded by the *ntaA* and *ntaB* genes

Component	Sequence
A	
NH_2	G A N K Q M N L G F L
921003[a]	GIGGCIGAIAAICAIATGAAIITIGG
ntaA	ATGGGTGCAAACAAGCAGATGAATCTGGGGTTCCTG
NtaA	M G A N K Q M N L G F L
B	
NH_2	A D Q I R S A T E G G
ntaB	ATGGCAGACCAAATTCGATCGGCAACGGAAGGCGGA
NtaB	M A D Q I R S A T E G G

[a] The sequence of oligonucleotide 921003 was derived from the N-terminal sequence of cA. At ambiguous positions, the nucleotide inosine (I) was incorporated.

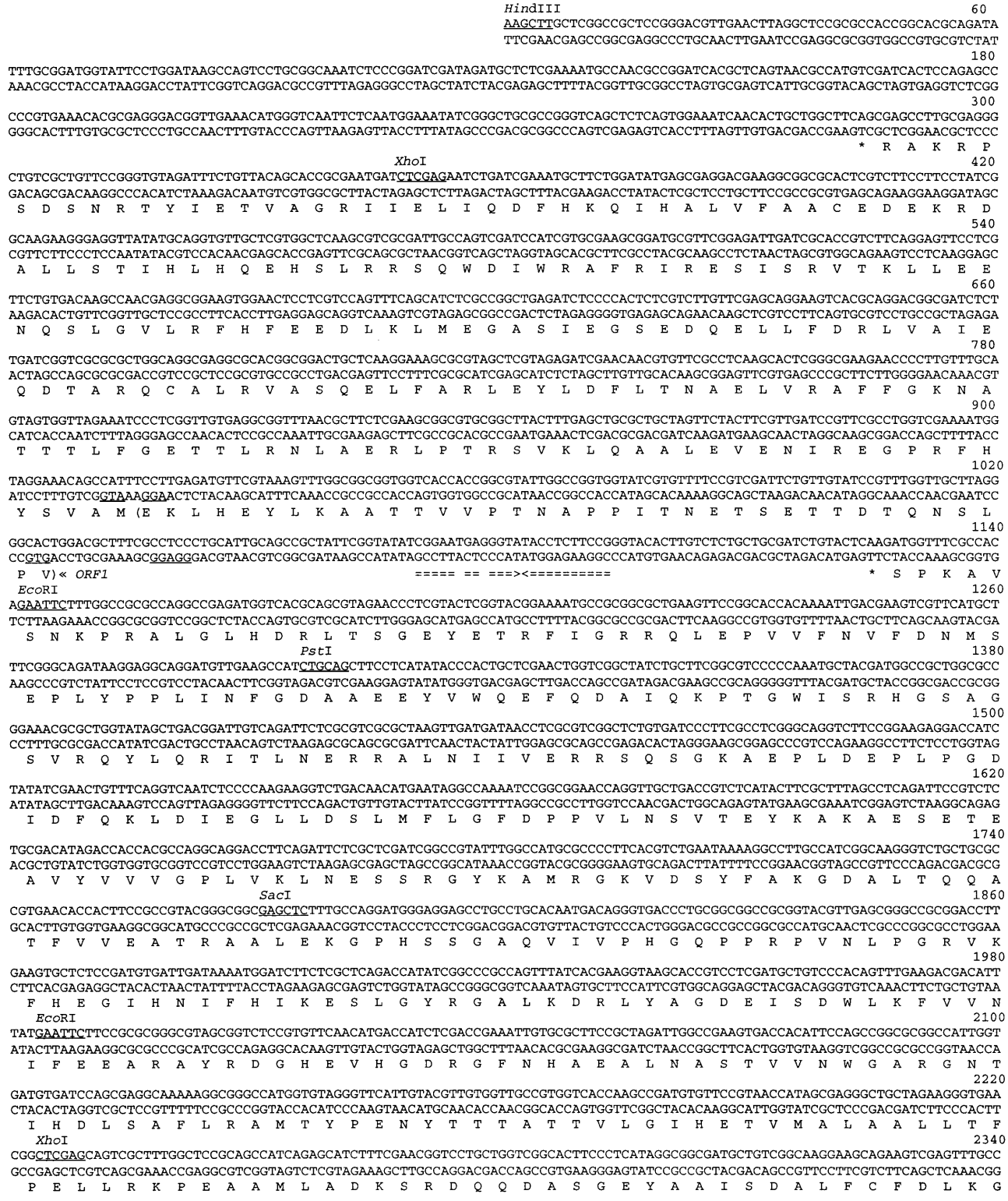

FIG. 3. Nucleotide sequence of the 5,931-bp *Hin*dIII-*Sal*I DNA fragment containing *ntaA*, *ntaB*, and ORF1. Relevant restriction sites, putative ribosome-binding sites (GAGG), and start codons (ATG) are underlined. Asterisks represent stop codons. Bases are numbered relative to the initial *Hin*dIII site. The predicted amino acid sequences encoded by *ntaA*, *ntaB*, and ORF1 are shown below the nucleotide sequence. The deduced directions of transcription are indicated by ⩾ and ⩽. The putative terminator structure between *ntaA* and ORF1 is depicted below the sequence.

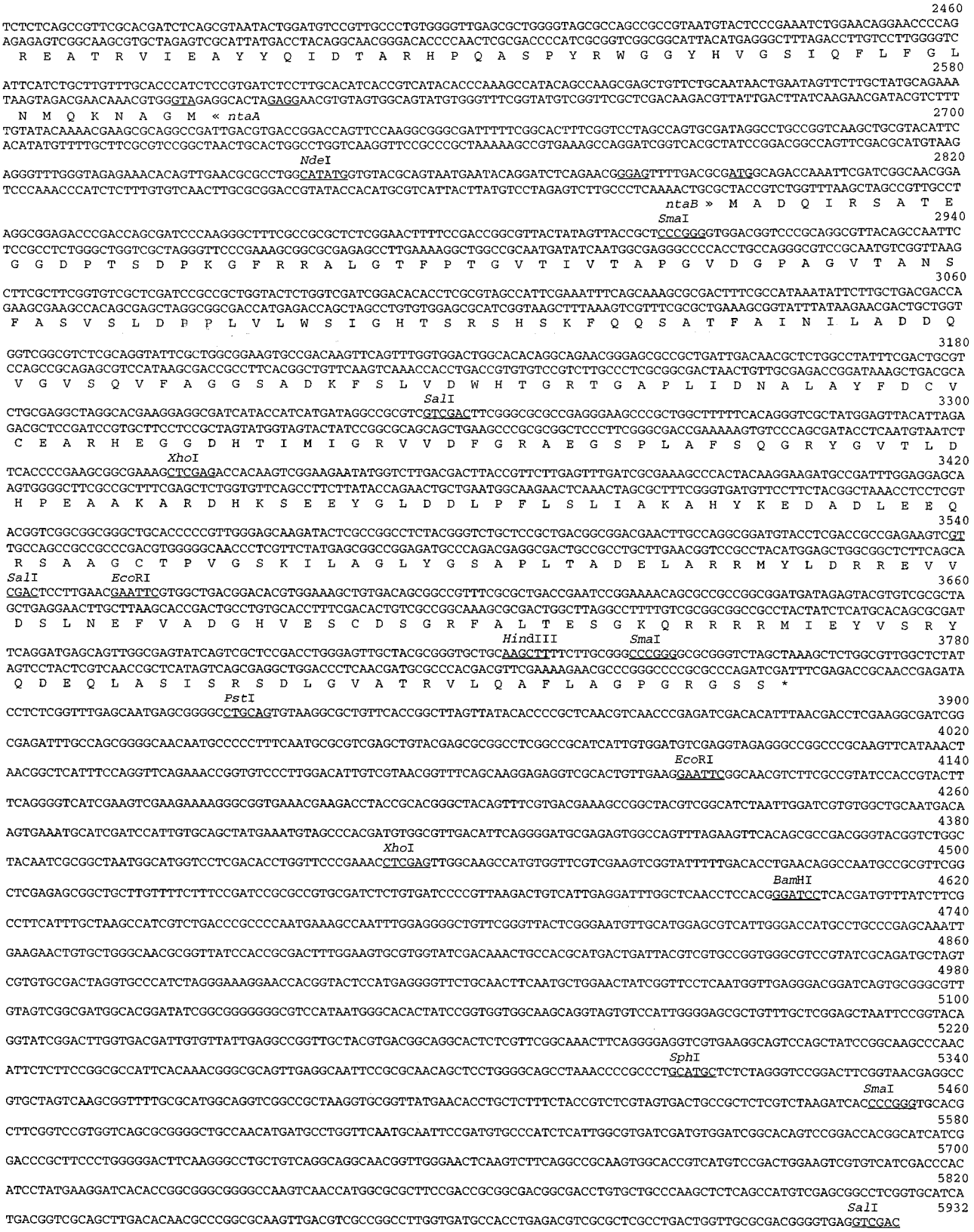

FIG. 3—*Continued.*

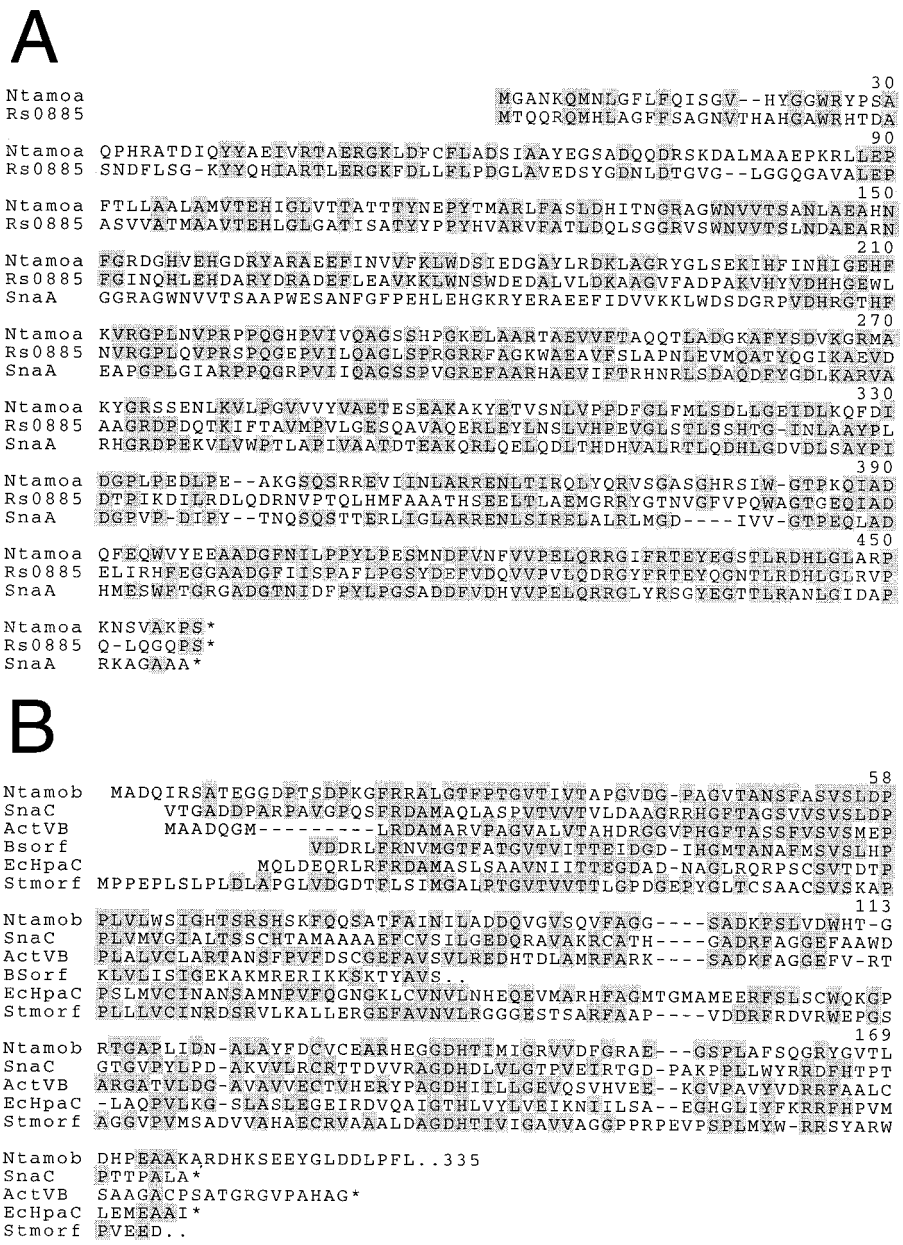

FIG. 4. FastA alignment of the predicted amino acid sequence of (A) NtaA (Ntamoa) from *C. heintzii* ATCC 29600 with that of SoxA (Rs0885) from *Rhodococcus* sp. strain IGTS8 (12) and with the C-terminal part of SnaA from *S. pristinaespiralis* (7). (B) Alignment of the N-terminal region of NtaB (Ntamob) with SnaC from *S. pristinaespiralis* (7), ActVB of *S. coelicolor* (20), partial peptide from an ORF next to PheA of *Bacillus stearothermophilus* (Bsorf) (31), HpaC from *E. coli* (EcHpaC) (36), and a partial peptide from an ORF next to the tetracycline self-defense gene of *S. aureofaciens* (Stmorf) (11).

GTG at position 1025 could function as such. This ORF1 might code for a protein of 210 (or 257) amino acids with a calculated size of 24.3 (or 28.3) kDa. No significant difference in codon usage was found between *ntaA*, *ntaB*, and ORF1. The GC content for these genes was 57 to 59%. Between the end of the *ntaA* gene and the start of the ORF1 at positions 1070 to 1093 (Fig. 3), we located a possible terminator structure with a stability of $\Delta G^{\circ}(25^{\circ}C) = -91.2$ kJ/mol as calculated by the method of Tinoco et al. (47).

Homology to other proteins. The deduced amino acid sequences of the three ORFs were compared with sequences of other proteins in the GenEMBL database. Not many other sequences with significant homology to NtaA or NtaB were found. NtaA appeared 40.1% identical to SoxA (12) in a 451-amino-acid overlap alignment (Fig. 4A). SoxA is a 50-kDa protein involved in the conversion of dibenzothiophene-5,5′-dioxide to an unidentified intermediate (possibly 2-hydroxybiphenyl sulfonic acid). In addition, NtaA was 49.2% identical in a 250-amino-acid C-terminal region to SnaA, one of the subunits of the *Streptomyces pristinaespiralis* pristamycin II_A synthase (7). NtaB showed homology in its N-terminal region to a number of proteins with oxidoreductase function, although these were substantially shorter than NtaB, and to a number of partially translated ORFs of unknown function (Fig. 4B). For example, NtaB showed 35.1% identity in 154 amino acids compared with ActRV of actinorhodin polyketide synthase from

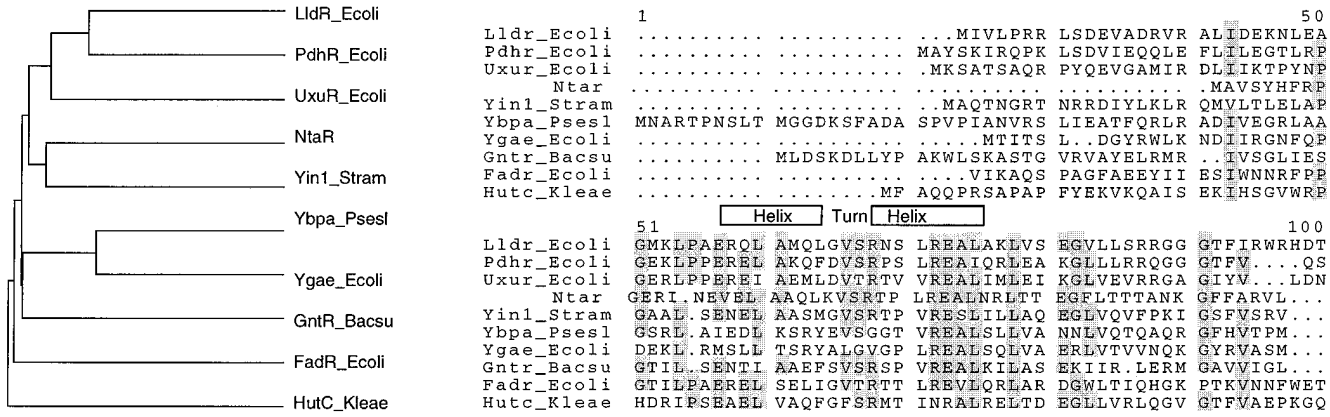

FIG. 5. Comparison of the N-terminal region of the predicted gene product of ORF1 with those of the uncharacterized ORFs Yin1_Stram.Swiss from *S. ambifaciens*, Orf0 (Ybpa_Psesl.Swiss) from *Pseudomonas* sp. strain LB400, and various GntR-type regulatory proteins: LctR (putative regulator of the L-lactate dehydrogenase operon of *E. coli*) (Lldr_Ecoli.Swiss) (16), PdhR (repressor of pyruvate dehydrogenase expression of *E. coli*) (Pdhr_Ecoli.Swiss) (28), HutC (repressor for histidine utilization of *Klebsiella aerogenes*) (Hutc_Kleae.Swiss) (43), GntR (repressor of the gluconate operon of *Bacillus subtilis*) (Gntr_Bacsu.Swiss) (24), UxuR of *E. coli* (Uxur_Ecoli.Swiss), YgaE of *E. coli* (Ygae_Ecoli.Swiss), and FadR (regulator of fatty acid metabolism of *E. coli*) (Fadr_Ecoli.Swiss) (15). The prediced helix-turn-helix motif is indicated above the aligned sequences. The left side shows a PILEUP clustering obtained from an alignment of the complete amino acid sequences. For example, the uncorrected distance between the ORF1 product of the *nta* cluster and Yin1_Stram.Swiss was calculated as 73.21 amino acid changes per 100.

Streptomyces coelicolor A3 (20) and 25.6% identity in 160 amino acids compared with HpaC from *E. coli* (36). Interestingly, from the same pristamycin synthesis system of *S. pristinaespiralis*, another protein (SnaC) exhibited significant homology to NtaB (32.5% in a 151-amino-acid overlap). SnaC functions as the oxidoreductase for the SnaAB-mediated synthesis of pristamycin (7). The *actRV* gene encodes a 18-kDa protein acting as a dimerase that joins two molecules of a late precursor of actinorhodin to produce the final antibiotic structure. It was also shown to functionally replace SnaC (7). HpaC is proposed to be a 19-kDa coupling protein enhancing activity of the 4-hydroxyphenylacetate 3-hydroxylase from *E. coli*. As the biochemical characterization of NtaB had previously indicated the presence of a weakly bound FMN, we tried to identify a consensus flavin-binding site in the primary sequence. However, neither the amino acid sequence of NtaB nor the aligned sequence of the oxidoreductases (Fig. 4B) contained one of the typical fingerprints (14), suggesting the presence of a novel type of flavin-binding site or a noncovalent binding of FMN to NtaB. Perhaps this C-terminal conserved region also contains an NADH-binding site.

The predicted gene product of ORF1 showed significant homology in a small region with the N-terminal helix-turn-helix motif of the GntR class of bacterial regulator proteins (Fig. 5). With two other polypeptides in the GenBank database, the ORF1 product showed significant overall homology: 26.3% identity in a 213-amino-acid overlap with an uncharacterized gene product from *Streptomyces ambifaciens* (Saunst) and 21.4% identity in a 192-amino-acid overlap with the product of ORF0 from the *Pseudomonas* sp. LB400 biphenyl dioxygenase operon (Fig. 5). We suspect that the gene product of ORF1 may be a regulator protein, perhaps involved in regulation of *ntaA* and *ntaB*, although we were not able to demonstrate its activity in *E. coli* (data not shown).

Expression of NTA monooxygenase in *E. coli*. The expression of *ntaA* in *E. coli* DH5α was tested by Western blotting of total cell extracts separated by SDS-PAGE and incubated with polyclonal antibodies against the purified cA of the NTA monooxygenase. Different plasmids with parts of the *ntaA* gene were constructed for this purpose (Fig. 2). Low expression of the *ntaA* gene was found independently of the orientation of the gene compared with the vector-located *lac* promoter (Fig. 6). Disruption of the *ntaA* gene such as in plasmids pNTA10, pNTA4, and pNTA5 caused loss of detectable expression of NtaA in Western blots. Similarly, low expression of the *ntaB* gene was detectable in *E. coli* DH5α from plasmid pNTA9 (data not shown). We decided to clone the *ntaA* and *ntaB* genes under control of the inducible T7 promoter in pET vectors to obtain a higher level of expression in *E. coli*. High levels of NtaA and NtaB were indeed obtained in *E. coli* BL21(DE3) harboring plasmid pNTA21 (*ntaA*) or pNTA17 (*ntaB*) upon induction with IPTG (Fig. 7). In cell extracts of *E. coli* BL21(DE3) harboring plasmid pNTA17, two strong protein bands were detected upon SDS-PAGE and on Western blots with anti-cB antibodies. One of these had a size larger than that predicted for NtaB. This probably results from an upstream start codon which is in frame with the N-terminal methionine at nucleotide 2791 (Fig. 3).

We then tried to reconstitute an active two-component NTA monooxygenase by mixing purified cA with a cell extract of *E. coli* BL21(DE3)(pNTA17) expressing NtaB. Activity of NTA monooxygenase was measured both as the consumption of NTA and as the formation of glyoxylate. A distinct increase in glyoxylate concentration was observed with a concomitant decrease in NTA concentration (Fig. 8). In contrast, the reconstitution of an active two-component NTA monooxygenase by mixing purified cB with cell extracts of *E. coli* BL21 (DE3)

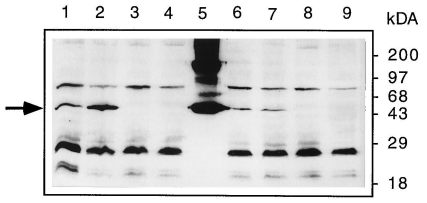

FIG. 6. Western blot analysis of NtaA expression in *E. coli* DH5α containing different plasmid constructs. Lanes: 1, total cell extract of *E. coli* DH5α(pNTA3); 2, pNTA6; 3, pNTA10; 4, pNTA4; 5, 4 μg of purified cA of NTA monooxygenase; 6, pNTA8; 7, pNTA7; 8, pNTA5; 9, pUC19. Relative migration of proteins with defined molecular masses is indicated on the right. The arrow on the left points to the NtaA protein.

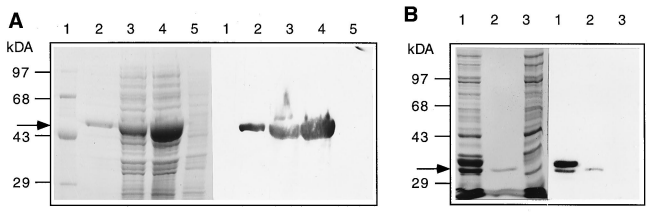

FIG. 7. Overexpression of *ntaA* (A) and *ntaB* (B) in *E. coli* BL21(DE3) total cell extracts. (A) Left, Coomassie blue-stained SDS-polyacrylamide gel. Lanes: 1, molecular mass markers; 2, 4 μg of purified cA of NTA monooxygenase; 3, *E. coli* BL21(DE3)(pNTA21); 4, same as lane 3; 5, *E. coli* BL21(DE3)(pET8c). The right part shows the corresponding Western blot immunostained with polyclonal antibodies against purified cA. (B) Left, SDS-polyacrylamide gel. Lanes: 1, *E. coli* BL21(DE3)(pNTA17); 2, 3 μg of purified cB of NTA monooxygenase; 3, *E. coli* BL21(DE3)(pET8c). The right part shows the corresponding Western blot immunostained with polyclonal antibodies against purified cB.

(pNTA21), expressing NtaA, was not successful. The specific NTA monooxygenase activities determined in these reconstitution experiments are given in Table 3; the assays included as a negative control purified cA mixed with cell extract of *E. coli* BL21(DE3)(pET8c) and as positive controls cell extract from NTA-grown *C. heintzii* ATCC 29600 and purified reconstituted NTA monooxygenase). These experiments demonstrated that NtaB overproduced in *E. coli* was functionally able to replace purified cB in the enzyme assay. However, NtaA overproduced in *E. coli* was functionally not able to replace purified cA in the enzyme assay. The reason for this is not known.

DISCUSSION

By using an oligonucleotide probe derived from the N-terminal sequence of purified cA, it was possible to isolate two overlapping DNA fragments from *C. heintzii* ATCC 29600 spanning 6.2 kb of DNA. The nucleotide sequence of this fragment contained three major ORFs. On the basis of the N-terminal amino acid sequences of the purified proteins, two of the ORFs could be assigned to NTA monooxygenase components A and B, respectively. Expression of these genes in *E. coli* resulted in proteins of the same size as the purified proteins, cross-reacting with anti-cA and anti-cB antibodies. Finally, reconstitution of NTA monooxygenase activity was possible by combining purified cA and *E. coli* cell extracts in which NtaB was overexpressed. Therefore, we propose to name these genes *ntaA* and *ntaB*, for components A and B, respectively, of NTA monooxygenase.

In previous work of our group, the NTA monooxygenase

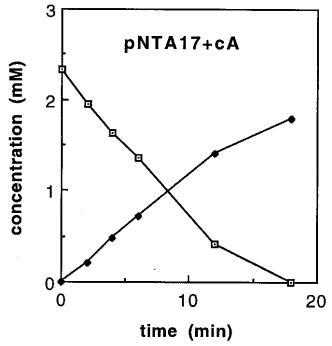

FIG. 8. NTA consumption (squares) and glyoxylate production (diamonds) during time in a mixture of purified cA of NTA monooxygenase and a cell extract of *E. coli* BL21(DE3)(pNTA17) expressing NtaB.

TABLE 3. Specific NTA monooxygenase activities

Strain in reaction mixture	Purified protein[a]	Sp act of NTA-Mo[b] (nmol of NTA · min^{-1} · mg of $protein^{-1}$)
None	cA and cB	875
C. heintzii ATCC 29600	None	76
E. coli BL21(DE3)(pET8c)	cA	<1
E. coli BL21(DE3)(pNTA17)	cA	306
E. coli BL21(DE3)(pNTA21)	cB	<1

[a] cA was used at a concentration of 53 μg · ml^{-1}; cB was used at a concentration of 38 μg · ml^{-1}.

[b] NTA monooxygenase (NTA-Mo) activity was measured as NTA disappearance.

from the obligately aerobic, gram-negative bacterium *C. heintzii* ATCC 29600 was purified and characterized (51). The enzyme consisted of two components which had to be purified separately, but by mixing the two components, it was possible to reconstitute a functionally active NTA monooxygenase complex. The reconstituted NTA monooxygenase catalyzed the stoichiometric oxidation of NTA to IDA, glyoxylate, and H_2O, and the reaction was dependent on molecular oxygen, FMN, $MgCl_2$, and NADH (Fig. 1). On its own, cB exhibited NTA-stimulated NADH-oxidizing activity, in which H_2O_2 was formed but not NTA was consumed. The role of cA is still unknown, but oxidation of NTA by cB was observed only in the presence of cA. Purified cB contained a weakly bound flavin, whereas no flavin was found in cA. Neither component contained iron, indicating that iron-sulfur clusters or cytochromes were absent.

We could not detect any consensus patterns in the amino acid sequence of NtaB which would indicate a flavin-binding site such as found in other redox proteins. According to Di Marco et al. (14), nicotinamide-flavin adenine dinucleotide enzymes can be classified into two discrete groups: the oxidoreductase group and the aromatic substrate-utilizing flavin monooxygenase group. Recently, Prieto and Garcia (36) proposed a new family of two-component aromatic hydroxylases, such as the 4-hydroxyphenylacetate 3-hydroxylase of *E. coli*, which might contain as yet uncharacterized flavin adenine dinucleotide- and NADH-binding sites. Therefore, it is possible that NtaB belongs to such a new subgroup with a novel type flavin-binding site, which could also contain the SnaC and ActRV proteins (7). Another possibility is that the loosely bound FMN is not covalently linked to NtaB and therefore no FMN binding site exists in NtaB.

Interestingly, *ntaA* and *ntaB* are divergently oriented, with an intergenic region of 307 bp. This is an unusual organization for genes whose products make up one enzyme complex. A distance of 307 bp between the translational start sites suggests a back-to-back arrangement of the promoters (4) such as for the *malEFG-malKlamB* operon of *E. coli* (5). However, we found little sequence similarity, with no typical −35/−10 or −24/−12 promoter sequence in this 307-bp region. When we aligned the upstream regions of *ntaA* and *ntaB* with themselves, though, we detected a region 30 bp upstream of the start codon of *ntaA* which was very similar to one lying 44 bp upstream of the start codon of *ntaB*. These sequences (with a consensus 5′TGGCXTXXGGTGTGTA3′) may represent promoter sequences or perhaps the binding sites for a regulatory protein.

Downstream of the *ntaA* gene, we found a third major ORF (ORF1). A possible terminator structure was observed between *ntaA* and ORF1, suggesting that ORF1 is independently transcribed from *ntaA*. The predicted gene product of ORF1 (24 or 28 kDa) has a DNA-binding motif (helix-turn-helix)

characteristic for the GntR family (27) at its N-terminal end (Fig. 5). The members of the GntR family are typically 27 to 30 kDa in size and act as repressors. The size of ORF1, the N-terminal helix-turn-helix motif, and its independent transcription from the *ntaA* and *ntaB* genes despite its close proximity to those genes suggest that ORF1 could code for a putative NTA regulator protein. We had observed previously that expression of NTA monooxygenase in *C. heintzii* ATCC 29600 is inducible when the cells are grown with NTA or IDA but not with a range of 20 other compounds (18, 50). This finding indicates that NTA and its first metabolite IDA are inducing compounds and that a regulatory system exists for expression of the NTA monooxygenase genes. We compared the consensus sequence occurring upstream of the *ntaA* and *ntaB* genes (TGGCXTXXGGTGTA) with the operator sequences of different GntR family members, such as GntR (ATACTTGTAT ACAAGTAT) (28), FadR (AGCTGGTCCGAYNTGTT) (38), and FarR (TGTATTA[A/T]TT) (37) but observed no obvious sequence similarity with any of these binding sites. It must be noted, however, that the DNA-binding sites of different GntR family members have little sequence homology in common.

The synthetic chelating agent NTA is the only known substrate for NTA monooxygenase, whereas several structurally similar compounds, such as *N*-methyl-IDA, *N*-acetamido-IDA, EDTA, and citrate, are not transformed by the enzyme (51). Perhaps uncharacterized natural substrates exist for NTA monooxygenase. Possible candidates could be naturally occurring aminohydroxypolycarboxylates, such as ethylenediaminedisuccinic acid (35) or rhizobactin (9). The enzyme complex may also be the result of a relatively recent evolutionary selective process for this xenobiotic compound. A comparison of the *ntaA* and *ntaB* gene sequence with other known sequences might elucidate some relationships. According to Harayama and Timmis (25), common ancestries for two proteins can be assumed if the following criteria are met: (i) they catalyze similar reactions, (ii) they have similar subunit molecular weights, (iii) their amino acid sequences can be aligned without the introduction of multiple gaps, and (iv) their amino acid identity is higher than 30%. The two proteins NtaA from *C. heintzii* ATCC 29600 and SoxA from *Rhodococcus* sp. strain IGTS8 (12) meet almost all of these criteria. NtaA and SnaA align only in their C-terminal parts (Fig. 4A). NtaA and SoxA have similar molecular masses of about 50 kDa. Their amino acid sequences can be aligned over the complete sequence of 451 amino acids with an identity of 40.1%. Whether the first criterion, the catalysis of similar reactions, can be fulfilled is not absolutely certain because the catalytic role of NtaA is still unknown. Two roles for NtaA can be envisaged: (i) cA could modify the active site on cB in a way such that NTA is accepted as a substrate and becomes hydroxylated or (ii) cA could be the component with the actual hydroxylating activity. SoxA catalyzes the conversion of dibenzothiophene-5,5′-dioxide to an unidentified intermediate (possibly 2-hydroxybiphenyl sulfonic acid). Therefore, it is possible that NtaA and SoxA catalyze an α-hydroxylation of the carbon atom next to the nitrogen or sulfur atom, although the structures of their substrates (NTA and dibenzothiophene-5,5′-dioxide) are very different. This is not uncommon, however. Recently two enzymes, mandelate racemase and muconate-lactonizing enzyme, which catalyze different chemical reactions but may have a common ancestor, were described (34). For NtaB, the situation is less clear. The predicted amino acid sequence of NtaB (36 kDa) has homologies of approximately 30% with the proteins ActRV, an 18-kDa putative dimerase of actinorhodin polyketide synthase from *S. coelicolor* A3 (20), HpaC, a 19-kDa coupling protein of the 4-hydroxyphenylacetate 3-hydroxylase from *E. coli* (36), and SnaC, an FMN oxidoreductase from *S. pristinaespiralis* (7). The proteins ActRV, SnaC, and HpaC are clearly much shorter than NtaB, yet they seem to catalyze comparable electron transfer reactions. The NtaB protein, about two times larger, apparently contains an extra domain which perhaps is needed for the specificity of the reaction with NTA. No homologies in this part of NtaB with other known protein sequences were found. The unusual back-to-back organization of the *ntaA* and *ntaB* genes may indicate that the genes *ntaA* and *ntaB* accidentally came together by DNA rearrangements as described for various aromatic pathway genes (52) and did not evolve jointly. Remarkably, the *snaA* and *snaC* genes, the only other system thus far identified in which two proteins involved in one biochemical reaction have homology with NtaA and NtaB, respectively, are separated by at least 24 kb on the genome of *S. pristinaespiralis* (7). A further interesting observation in this respect was that upon hybridization of genomic DNA from two other NTA-degrading isolates, *C. asaccharovorans* TE2 and *C. heintzii* TE6, with *ntaA* or *ntaB*, the *ntaA* gene appeared well conserved, whereas the *ntaB* gene was detectable in not at all TE6 and to only a low level in strain TE2 (32). This finding suggests that the enzyme activity of NtaB may be fulfilled by other proteins, since NTA monooxygenase activity was detected in all of these strains (50).

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ADDENDUM

During review of the manuscript, a sequence essentially the same as that of *ntaA*, *ntaB*, and ORF1 from *C. heintzii* ATCC 29600 appeared in GenBank (submitted by Y. Xu et al., accession number L49438).

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