A plasmid encoding enzymes for nylon oligomer degradation: nucleotide sequence and analysis of pOAD2

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The entire nucleotide sequence of nylon oligomer degradative plasmid pOAD2 from *Flavobacterium* sp. KI723T1 was determined. pOAD2 comprises 45 519 bp, with a 66.6 mol% G + C content. The precise loci of the four nylon oligomer degradation genes, namely *nylA* (6-aminohexanoate-cyclic-dimer hydrolase gene), *nylB* (6-aminohexanoate-dimer hydrolase), *nylB'* (a gene having 88% homology to *nylB*) and *nylC* (endo-type 6-aminohexanoate oligomer hydrolase), and five IS6100 elements were identified on this plasmid. Comparison of the sequence of pOAD2 with those in the GenBank and EMBL databases revealed that the deduced amino acid sequences from eight regions of pOAD2 had significant similarity with the sequences of gene products such as *oppA–F* (oligopeptide permeases), *ftsX* (filamentation temperature sensitive), *penDE* (isopenicillin N-acyltransferase) and *rep* (plasmid incompatibility). A functional map of pOAD2 is presented.

Keywords: nylon oligomer, degradative plasmid, enzyme evolution

INTRODUCTION

Research on the microbial degradation of xenobiotic compounds produced by chemical industries is not only important for the environmental sciences, but is also interesting with regard to enzyme evolution. We have been studying the degradation of 6-aminohexanoate oligomers (nylon oligomers found as by-products of the nylon-6 industry) by *Flavobacterium* sp. strains as a model of how micro-organisms have evolved specific enzymes that degrade xenobiotic compounds. Previous biochemical studies have revealed that three enzymes produced by *Flavobacterium* sp. KI723T1, 6-aminohexanoate-cyclic-dimer hydrolase (EI) (Kinoshita *et al.*, 1977), 6-aminohexanoate-dimer hydrolase (EII) (Kinoshita *et al.*, 1981), and endo-type 6-aminohexanoate oligomer hydrolase (EIII) (Negoro *et al.*, 1992), are responsible for the degradation of 6-aminohexanoate oligomers. Cloning and sequencing analyses have shown that the EI (*nylA*), EII (*nylB*) and EIII (*nylC*) genes are located on pOAD2, one

of three plasmids harboured by *Flavobacterium* sp. KI723T1 (Negoro *et al.*, 1980, 1983), and that these genes are encoded by ORFs of 1479 bp (*nylA*), 1176 bp (*nylB*) and 1065 bp (*nylC*). Enzymes EI (P-EI) and EII (P-EII) in *Pseudomonas* sp. NK87 (P-*nylA* and P-*nylB* genes) are plasmid-encoded (Kanagawa *et al.*, 1989), and the amino acid sequence identities between F-EI and P-EI and between F-EII and P-EII are 99% and 35%, respectively (Kanagawa *et al.*, 1993; Tsuchiya *et al.*, 1989).

Previous Southern hybridization experiments have revealed the presence of at least two repeated sequences (RS-I and RS-II) on pOAD2. The RS-I regions were found at five loci (Negoro *et al.*, 1983). Four of the five RS-Is (RS-I_A, I_C, I_D and I_E) are identical to IS6100 (880 bp) initially found in *Mycobacterium fortuitum*, while the remaining one (RS-I_B) contains an additional 420 bp, which is a repeated portion of the IS6100 element, at the 3'-terminus (Kato *et al.*, 1994). RS-II is found at two loci, namely RS-II_A, which contains the *nylB* gene, and RS-II_B, which contains the homologous *EII'* gene (*nylB'*) (Okada *et al.*, 1983). The two genes are identical in size and encode peptides of 392 amino acids differing only in 46 residues (Okada *et al.*, 1983).

Although the nucleotide sequences of the *nyl* genes and IS6100 have been determined, they comprise only 10% of

Abbreviations: EI, 6-aminohexanoate-cyclic-dimer hydrolase; EII, 6-aminohexanoate-dimer hydrolase; EIII, endo-type 6-aminohexanoate oligomer hydrolase; NSF, non-stop frame; RS, repeated sequence.

The GenBank/EMBL/DBJ accession number for the sequence reported in this paper is D26094.

the total length of pOAD2. In this paper, we describe the nucleotide sequence of pOAD2, and construct a functional map of the plasmid based on the homology analyses.

METHODS

Bacterial strains and plasmids. *Flavobacterium* sp. KI723T1 was used as a strain for preparing pOAD2 (Negoro *et al.*, 1980). Three plasmids which contain *Hind*III fragments of pOAD2, i.e. pNDH16 (A+D+F fragments), pNDH56 (B+E fragments) and pNDH5 (C fragments), were constructed as previously described using pBR322 as the vector (Negoro *et al.*, 1983) (Fig. 1). Plasmids pBR322 (Bolivar *et al.*, 1977), pBluescript SK⁺ (Stratagene Cloning Systems), M13mp18 and M13mp19 (Yanisch-Perron *et al.*, 1985) were also used as vectors.

DNA manipulations. Plasmid DNA of *Escherichia coli* was prepared by the alkaline extraction method (Birnboim & Doly, 1979) and purified by CsCl-ethidium bromide density-gradient centrifugation (Sambrook *et al.*, 1989). Plasmids of *Flavobacterium* strains were similarly prepared with some modifications (Negoro *et al.*, 1980). To separate pOAD2 from the other two plasmids (pOAD11 and pOAD3) in the KI723T1 strain, the plasmid mixtures obtained by the CsCl-ethidium bromide density-gradient centrifugation were digested with *Xba*I, and the DNAs were re-fractionated by density-gradient centrifugation. The pOAD2 has no *Xba*I site and hence is maintained as covalently closed circular DNA. The other two plasmids are converted to linear fragments after *Xba*I digestion. Hybrid plasmids pNDE1 and pNDE2 were constructed as follows: pOAD2 was digested with *Eco*RI, and the digested samples were subjected to agarose gel (1%, w/v) electrophoresis (Sambrook *et al.*, 1989). The *Eco*RI-A (24 kb) and -B (15 kb) fragments were recovered (Fig. 1), and these fragments were cloned into the *Eco*RI site of pBR322 (Bolivar *et al.*, 1977). Restriction endonuclease digestion, ligation and transformation were done by conventional methods (Sambrook *et al.*, 1989). Recovery of the DNA fragments from the agarose gel was performed by using a Gene Clean kit (Bio101) (Vogelstein & Gillespie, 1979).

Sequencing strategy. pNDH5, pNDH16 and pNDH56 were used as initial materials for sequencing. Sub-fragments from pNDH5, pNDH16 and pNDH56 were cloned into M13mp18 and M13mp19. To confirm that the inserted DNA fragments were not altered through cloning experiments, pOAD2 from the original KI723T1 strain was first digested with *Hind*III, and each fragment was analysed by agarose gel electrophoresis. The patterns of various restriction enzyme digestions of the fragments recovered from pOAD2 were confirmed to be the same as those from pNDH plasmids. To sequence the region including the *Hind*III sites, sub-fragments containing the *Hind*III site at positions 0/45.5, 13.5, 14.7, 16.8, 28.8 or 37.9 kb were isolated from pNDE1 and pNDE2, and cloned into pBluescriptII. Sequences of both strands of the whole pOAD2 region were determined by the dideoxy chain-termination method (Sanger *et al.*, 1977). Sequences including terminal regions in sub-fragments overlapped with adjacent sequences.

RESULTS AND DISCUSSION

Identification of the nucleotide sequence of pOAD2

The entire sequence of the nylon oligomer degradative plasmid pOAD2 comprised 45519 bp. pOAD2 contained

Fig. 1. (a) Location of the NSFs on pOAD2. The three clockwise NSFs (NS1, NS2 and NS3) and three counterclockwise NSFs (NA1, NA2 and NA3) are shown as broken circles. Bold numbers represent the 34 clockwise NSFs and the outlined numbers represent the 37 counterclockwise NSFs. The regions which have significant homology with some proteins registered in the database are shown as heavy boxes. The location of the six *Hind*III fragments (A-F) and the five *Eco*RI fragments (A-E) are shown in the closed circles. (b) Location of the seven NSFs in the 2.9–11 kb region of pOAD2. The regions showing homology with the *opp* gene cluster are shown as heavy boxes (see Fig. 2). The promoter region essential for the expression of the *nyiB* in *E. coli* (Okazaki *et al.*, 1988) is represented by an arrow. Identity between the *opp* genes and the region on pOAD2 is also shown.

876 termination codons in the three frames of clockwise direction and 778 termination codons in the three frames of counterclockwise direction (Fig. 1). The clockwise strand contained 7108 adenines, 15514 guanines, 14812 cytosines and 8085 thymines. The G+C content was 66.6%. The plasmid size was in good agreement with that estimated by electron microscopy or by agarose gel electrophoresis, i.e. 43.6 kb (Negoro *et al.*, 1980).

Table 1. Identified gene products found on pOAD2

NSF	Location of NSF	Identified element or gene	Location of the gene* (Init. codon-term. codon)
NS2-1	2-802	RS-I _A (IS6100-transposase)	41-805
NS2-14	14327-15127	RS-I _B (IS6100-transposase)†	14366-15130
NS3-18	17871-18671	RS-I _C (IS6100-transposase)	17910-18674
NA1-28	29212-30012	RS-I _D (IS6100-transposase)	29973-29209
NA3-34	38754-39554	RS-I _E (IS6100-transposase)	39515-38751
NA3-4	2814-4601	<i>nylB</i> (EII)	3989-2811
NA3-7	4605-6566	<i>nylC</i> (EIII)	5669-4602
NS2-15	15641-17044	<i>nylB'</i> (EII')	15869-17047
NS2-24	30338-31837	<i>nylA</i> (EI)	30359-31840

* Location of the gene indicates the position from the initiation Met codon (init.) to the termination codon (term.).

† In RS-I_B, the 420 bp region at the 3' terminus (location: 15185-15604) was a repeated portion of the IS6100 element.

Table 2. Deduced gene product showing significant homology to extant protein

NSF	Location of NSF	Gene	Origin	Score*	Number of amino acids†	Homology (%)	Reference
NA2-9	5669-7402	<i>oppF</i>	<i>Bacillus subtilis</i>	68.2	261	43.7	Perego <i>et al.</i> (1991)
NA1-10	6439-8193	<i>oppD</i>	<i>Bacillus subtilis</i>	59.0	259	39.0	Perego <i>et al.</i> (1991)
NA3-11	7257-10856	<i>oppC</i>	<i>Bacillus subtilis</i>	51.1	273	29.3	Perego <i>et al.</i> (1991)
NA2-12	8126-9100	<i>oppB</i>	<i>Bacillus subtilis</i>	27.3	316	27.5	Perego <i>et al.</i> (1991)
NA2-13	9104-10861	<i>oppA</i>	<i>Bacillus subtilis</i>	18.5	349	19.2	Perego <i>et al.</i> (1991)
NS3-2	885-1958	<i>ftsX</i>	<i>E. coli</i>	18.4	293	22.2	Gill <i>et al.</i> (1986)
NA3-15	11259-13058	<i>penDE</i>	<i>Aspergillus nidulans</i>	31.8	336	27.7	Tobin <i>et al.</i> (1990)
			<i>Penicillium chrysogenum</i>	26.7	337	26.7	Tobin <i>et al.</i> (1990)
NA1-20	20026-20841	<i>repA</i>	<i>Agrobacterium tumefaciens</i> (pTiB6S3)	20.4	173	28.3	Tabata <i>et al.</i> (1989)
		<i>sopA</i>	<i>E. coli</i> (F)	17.1	254	24.8	Mori <i>et al.</i> (1986)
		<i>parA</i>	<i>E. coli</i> (P1 phage)	15.5	127	33.9	Davis <i>et al.</i> (1992)
		<i>incC</i>	<i>E. coli</i> (RK2)	17.1	225	28.0	Thomas & Smith (1986)

* The level of homology was judged by a statistical method (see text). The score was expressed as the ratio of the homology score in the library of the random sequences to their corresponding SD.

† Numbers of amino acid residues in the homologous region. For NA2-9, 1-10, 3-11, 2-12 and 2-13, regions homologous with Opp proteins are shown in Fig. 2.

Functional map of pOAD2 based on the homology analysis

The ORF is generally used as a discrete unit for homology searches. However, an ORF is defined by the presence of a major initiation codon (ATG) or some minor initiation codons, such as GTG and TTG, and termination codons (TAA, TAG and TGA). Since this definition may unnecessarily restrict searches for homology, we compared the sequences of pOAD2 and those in the database with non-stop frames (NSFs), i.e. coding regions flanked by one termination codon and its successive termination codon on the sequence (Yomo *et al.*, 1992). A data library of amino acid sequences covering the total length of the

registered sequences in the sense and antisense strands from the DNA database (GenBank release 71; EMBL release 30) was initially constructed. All the amino acid sequences of the 71 NSFs found in pOAD2 were then aligned with those in the constructed data library by using the computer program FLAT in DDBJ (Pearson & Lipman, 1988). Random sequences were first generated from the same amino acid composition of the found NSFs. The level of homology between these random sequences and the selected sequences from the data library was judged by a statistical method (program RDF2 of FLAT in DDBJ) (Pearson & Lipman, 1988).

Fig. 1 shows the location of NSFs with more than 600 bp.

(a)
NA2-9 GARRAAGGLHENAGRGHARPRGSRGEGDGMMSAKTTTPVLELRDVSVAFTSGAGAKRRTVR
OppF MNÉLTÉKLÉIKHLKQHFV----TPRGTVK
323
AMNAVTELEVAPETGLGVSGSGKTTTASVALGLKRPDSGEVLLQ-SPFTSSRRAN--
AVDDLSFDIYKGETLGLVSGSGCKSTGRSIRLRYEATDGEVLNGENVHGRSKRKL
--AGKLQAVLQHPHWSLNPRKVGESVREPLTVARRDLGRSEQRQVTEMLEQVGLNPAF
EFNRKMQMI PQDPYASLNPRMTVADI AEGLDIHLKAKTKKERMQRVHELLETVGLNKEH
AERYPHELGGQRQVRVARALVTEPRFVDFEASVALDVAVQMQLALIRELQAEHRFG
ANRYPHFSGGQRQRI GIALALAVDPEFI ADEPISALDVSIAQVVLMLKELQKEXGLT
ALFISHDLGAVQRVADRVAVLFRGDLVETATDEFFRPNRHPYSQTLLEAL
YLFIAHDLMSVKYISDRIGVMYFGKLVELAPADELYENLHPYTKSLLSAIPLPDPDYER
578
(b)
NA1-10 PLHQPHRRHDARSARSARDKEPRMTANAPAPVSVLTGFSAEYRTRLGVTRALDGLDLDV
OppD MIRVTRLLÉVKDLAISFKTYGGEVQAIRGVNFHL
320
ARGELLAIVGESGSGKSTVSAIGRLP--RACHVTSQSVHVLGRDLADLAPDAIRALRRE
DKGETLAI VGESGSGKSVTSQAIMKLI PMPGYFKRGEILFEKGLDVLPLSEKEMQNVGRK
SLGFI PQDPIASLDPTMRIRRLQELAL---RPLGQVPDPGSLAQLLESVRILEPERVRL
EIGMIFQDPTSLNPTMKVQKQITVLFKHEKISKEAKKRAVLELVGIMPKEKRVNQ
YPEVSGGMAQRIALITMAREPEILIADEPTASLDAQVRDEILTLIVDLVQRSGASLIW
FPEHFGMRQVRVIALMALAANPKLLIADEPTTALDVTIQAQILEMKDLQKKIDTSIF
ISHDLAGVRRWCDRVVMHQRIVEEGPTARVLEPRADYTRTLVAAMPGRADLAAKETE
ITHDLGVVANVADRVAVMYAGQIVETGVDEIFYDPRHPYTWGLASMPTESSGEEELT
574
(c)
NA3-11 PHHERARPARRRHREGRIMTYSPLSKKKRAVKTTPRDLAFIAACALGLIVLVAVAA
OppC MQNIPKMFEPAAANAGDAEKISKSLKSLWKDÄHLFRSÑKLAHVGLIIIVLILMAIFA
923
AVIP--FAGDPAKIVGTRLSPPSAAYPLGTDALGRSVLARLEGRTRTLVLSTLAVVCTAV
PMFSRYDYSTTNLÄNÄ--KPPSKDHVFGTDLDGRDIFVRTWVGARISIFIGVAAVLDLL
ISVILGLIAGYAGQWAREVIMRLSDVLVSFSPISVLAAILAAVVGPOQAALVSSIVLVTV
IGVWGSISGFRGGTDEIMMRIDILWAVPSLLMVILLMVLPKGLFTIITAMTITGW
LMTRMVSAAAMSVAKRDFVTYAKISGVSAPIVILFRHLLPNVSGTIAVQGTYSALVGLVE
NMARIVRGQVLQKNQÉYVLAŠQTLGAKTSRLFLKHI VPNAMGSLVMTLITVPTAIFTE
GGSLFGLYGVQPPRSSLGLLIQEGGSMYLAQWLLFIPAAVLVVAISINLIQDTRMDRL
ÄFLSYLGLVPAPLÄSAGTMSDGLPÄLYYPWRFPFAGFICITMFQFNVDGLRDLAL
1194
DPRETRSLV
DPKLRK
(d)
NA2-12 FRMMQILRWSAGRVMSALITLGLVSLIFVAIRLMPGSYEDIIILGPLASAEERQLLAEAY
OppB MLKYIGRRIVYMIITLFVIVTVTFFLMQAAPGG--PFSGEKKLPPEIEANLNAHY
6
GLNQPIFVQYFAWIGQVLGTGLTSTVYTSQSVSGEMLER-IPVTATLTIMAMAVTLVGV
GLDKPLPVSYLKSVMWMDPFSKYKQSVNDLISSGFPVPSFTLGAEAILLALGLGV
PLGIWTVGRSGAKQGGAGRVVSSIGISVPEFVLGSIVIFLFSQFSLGLKVGFGFASITGT
LFGVIAÄLYHNKQDYTVÄ--ILTIFGISVPSFIMAÄVLQYVFS----MKLGLFPVAG--
FWPTLGAALLPALVLSVFCVAATARTTRDAVMGVLEPHITAARVARGETTRHVRHHLR
-WDSWAYTFLPSIALASMPMAFIARLSRŠMIÉVNSDYIRTAKAKGLSRPALTVRHAIR
NAGIPILTLGTITAYLLGGAVIETLFLNPLGSGPMVTGLGRDRFAVQATVLFAAAVF
NALLPVVYMGPMMAQVLTGSPIETIFGIPGLGAHFVNSITNRDITVIMGVTVFVSIL
320
VVLSLVLDLITSALDPRVAVTGKAGS
LLCVLIVDVLYGIIDPRIKLSKAKKGA
(e)
NA2-13 RALTVDVVGSLERQVASQSVWSGQMGPFVSEATQDDQVTVKLGSPYAPFLAALANTPA
OppA PNNESQYAYQLYIKGAEAAANTGKSLDDVAVKAVNDKTLKVELNPN--TPYFTTETAFYT
204
AILPMQEVNDGSDVLTITM---LGTQPYLVADHRQDESWTFTANPDWHGGDNLAQVTE
YMPIEKIÄEKKNKWNNTAGDDYVNGPFKMTÄWKHSŠITLÉKNQYVWDKÉVKKKID
LQIVDQESTRAALREGS---AGMA--NFVNIDSLSQLGGNEGLQIVKQTSQDLYVVLVNS
VMVINNNTELKKFQAGELDWAGMPLQLPTESLPTLKKDŠSLHV--ÉPIAGVYVYKFN
QKPDSPNLNDQDVFIAINTAIDRQAIADIVFAGETTP-TGITPSNLPG-----ACLVD-E
É--ÄKPLDNVNIRKALTYSLDRQSI VKNVTQGEQMPAMAAPPTMKGFEDNKEGYFKDND
LPSEQAÄIÄDARATIEETGAGDVELDLIVNTLEPVMAQIAQLVQQQL-ÄEIGTVRIEQV
VKTÄKÉYLEKGLKEMŠKASDLPKIKSYNTDÄHAKIAQAVQEMWKKNLGVDVELDŠS
DTATYNERVFSAPQDFDLS--ISWFAGYSDP-SMVTKWNNEQAFNVGFTGVHEDNLAL
ÉWNVYIDKL---HŠQDYQIGRNGWLGDFNDPINFLÉLFRDKNGGNDTGWE--NPÉFKKL
533
IAEGASETDPDARAEVITRLCASADEYAEVMPVLRHPSIIGFDTTAVSPTIQSNEGYNGI
LNQŠQTEKTKRAELLKKAEGIFIDEMPVAPIFYFTDTWQDENLKGVMIPGTGEVYFR

There were 34 NSF's on the three clockwise frames (NS2-1 to NS3-34) and 37 NSF's on the three counterclockwise frames (NA3-1 to NA2-37). Since almost all pOAD2 regions include at least one NSF, these defined NSF's were therefore used as primary discrete units for the homology search.

Among the 71 NSF's, nine NSF's could be identified as *nylA*, *nylB*, *nylB'*, *nylC*, and five *IS6100* elements (Table 1). In addition, there were eight NSF's that had high similarity with sequences in the database (Table 2). The regions having significant similarity in the database were the *opp* gene cluster (oligopeptide permease), *rep* (replication protein), *penDE* (isopenicillin *N*-acyltransferase) and *ftsX* (heat-shock response and cell division). The remaining 54 NSF's were found not to have a significant level of similarity with any sequences in the database.

We have reported that the expression of the *nylB* gene is controlled by a region approximately 6 kb away from the *nylB* gene (Okazaki *et al.*, 1988). Interestingly, within this 6 kb region, a *nylC* gene was found upstream. Moreover, by Northern hybridization experiments using the *nylB* probe, 7 kb and 9 kb mRNA strands were detected (Zhou *et al.*, 1990). Thus, *nylB* and *nylC* seem to constitute an operon with other unknown gene(s) (Zhou *et al.*, 1990). After sequencing pOAD2 and a homology search, we found that in between the promoter region and the *nylC* gene there is a DNA region homologous to the oligopeptide uptake (*opp*) gene clusters (Alloing *et al.*, 1990; Hiles *et al.*, 1987; Perego *et al.*, 1991; Rudner *et al.*, 1991). Five of the NSF's (NA2-9, NA1-10, NA3-11, NA2-12 and NA2-13) had significant homology (44–19%) with the *opp* genes (Figs 1b and 2, Table 2). OppD and OppF have 44.4% homology. Interestingly, 34% identity was found between NA2-9 and NA1-10 (Fig. 3). Since the N-terminus of NA2-13 is only 5 bp downstream of the putative -10 region (TACCGT, position 10872–10867), it is likely that these five genes are cotranscribed from this common promoter.

Oligopeptide permease in *Bacillus subtilis* is considered to constitute a complex of ATPase, inner-membrane protein and binding protein (Perego *et al.*, 1991). OppF and OppD, which have the ATPase activity, are localized in the cytoplasm, while OppB and OppC are membrane proteins. OppA is located on the cell surface and is assumed to be the initial binding site for substrates. Similar systems are also found in *Salmonella typhimurium* strains (Hiles *et al.*, 1987). Therefore, the low homology between NA2-13 and OppA suggests that divergence between the sequences in OppA may reflect major

Fig. 2. Alignment of amino acid sequences showing the identity between the NSF's and the *opp* gene cluster. (a) NA2-9 and OppF; (b) NA1-10 and OppD; (c) NA3-11 and OppC; (d) NA2-12 and OppB; (e) NA2-13 and OppA. Amino acids are represented with one-letter codes. Identical amino acids and analogous amino acids are marked by asterisks and dots, respectively. Numbers shown above the sequences indicate the position of the amino acid residues from the N-terminal of the NSF. Gaps are shown as dashes.

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NA2-9  MSAKTTPVLRLRDVSVAFSTGAGAKRRTVRAMNAVTLVAPGETLGLVSGSGSKTT-TA
NA1-10  APVVSLTGFSÄYRTRRGVT----RALDGI DLVARGELLÄIVGESGSGKSTVSQ

SVALGLKRP---DSGEVLLLGSPFTRSRANAGKL-QAVLQHPHWSLNPRKVGESVREP
ÄIGRMLPRACHVTSGSVHVLRDLADLAPDAIRALRRSLGFI PQDPIASLDPTMRIRRQ

LTVARRDLRSEQRQRVTEMLQV-GLNPFAFER-YPHLSGGQRQRVSARALVTEPRF
* * * * *
LÄLALRPLGGPVDPGSLÄQLLESVRI LEPÄRVLRLYPHEVSGGMAQRIÄIÄLTÄREPEI

IVFDEAVSALDVAVQMQLÄLÄRELQAEHRFGALFI SHDLGAVQRVADRVAVLFRGDLVE
* * * * *
LÄADEPTÄSLDÄQVRDEILTLIVDLVQSRGASLÄIWSHDLGAVRRWCÄRVVMHÄGRIVE

TAATDEFFRNPRHPYSQTLLÄÄL
ÄGPTÄRVLDÄPRÄDÄYTRTLVÄÄMP

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Fig. 3. Alignment of sequences showing identity between NA2-9 and NA1-10 proteins. The region of NA2-9 homologous with the OppF is aligned with the region of NA1-10 homologous with OppD (see Fig. 2). The identical amino acids and analogous amino acids are marked by asterisks and dots, respectively. Gaps are shown as dashes.

Fig. 4. Deduced functional map of pOAD2. Heavy boxes represent the regions encoding the identified gene products; i.e. four nylon oligomer degradation genes (*nylA*, *nylB*, *nylB'* and *nylC*) and IS6100 sequences appearing five times on the plasmid. Open boxes represent the DNA regions which have significant homology with the sequences of *oppA*, *oppB*, *oppC*, *oppD*, *oppF*, *ftsX*, *penDE* and *rep* genes. Arrows indicate the direction of gene transcription.

differences in substrate specificity. Since nylon oligomers and oligopeptides are analogous compounds in which monomeric units having an amino group and a carboxy group are combined by amide bonds, the gene clusters on pOAD2 might be involved in the transport of nylon oligomers.

NS3-2 had 22.2% similarity with *ftsX*, which is involved in the heat-shock response and affects cell division in *E. coli* (Gill *et al.*, 1986). The *ftsX* gene constitutes an operon with *ftsE* (Gill *et al.*, 1986), and *ftsE* and *oppD* have sequence similarity (Gill *et al.*, 1986). NA1-20 was homologous to several genes responsible for plasmid replication, such as *repA* from *Agrobacterium tumefaciens* plasmid pTiB6S3 (Tabata *et al.*, 1989), *incC* of the broad host range plasmid RK2 (Thomas & Smith, 1986), *sopA* of the *E. coli* mini-F plasmid (Mori *et al.*, 1986), and *parA* of *E. coli* P1 phage (Davis *et al.*, 1992) (Table 2). Since

there is no other DNA region which has high similarity to the gene responsible for replication, it is likely that NA1-20 is responsible for plasmid replication. NA3-15 had 28% identity to isopenicillin N-acyltransferase (*penDE*) from *Aspergillus nidulans* and *Penicillium chrysogenum* (Alvarez *et al.*, 1993; Tobin *et al.*, 1990).

Fig. 4 illustrates the functional map of pOAD2, though the biological function of most gene products has not yet been confirmed. The precise loci of the nine regions having identified functions (four *nyl* genes and five IS6100 elements) and the eight regions having significant similarity to the genes registered in databases are shown on the map. However, 54 NSF's remain to be identified, and have no similarity to sequences in the database. As additional sequences are registered in the database, other genes might be identified. The complete nucleotide sequence of this plasmid can serve as the basis for further biochemical, genetical and evolutionary studies on pOAD2, which will lead to a more basic understanding of microbial adaptation towards a xenobiotic compound and of the evolution of new genes such as those responsible for degradation of xenobiotic compounds.

REFERENCES

- Alloing, G., Trombe, M.-C. & Claverys, J.-P. (1990). The *ami* locus of the gram-positive bacterium *Streptococcus pneumoniae* is similar to binding protein-dependent transport operons of gram-negative bacteria. *Mol Microbiol* 4, 633-644.
- Alvarez, E., Meesschaert, B., Montenegro, E., Gutierrez, S., Diez, B., Barredo, J. L. & Martin, J. F. (1993). The isopenicillin-N acyltransferase and isopenicillin-N amidohydrolase, 6-amino-penicillanic acid acyltransferase and penicillin amidase activities, all of which are encoded by the single *penDE* gene. *Eur J Biochem* 215, 323-332.
- Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* 7, 1513-1523.
- Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Crosa, J. H. & Falkow, S. (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* 2, 95-113.
- Davis, M. A., Martin, K. A. & Austin, S. J. (1992). Biochemical activities of the *parA* partition protein of the P1 plasmid. *Mol Microbiol* 6, 1141-1147.
- Gill, D. R., Hatfull, G. F. & Salmond, G. P. (1986). A new cell division operon in *Escherichia coli*. *Mol & Gen Genet* 205, 134-145.
- Hiles, I. D., Gallagher, M. P., Jamieson, D. J. & Higgins, C. F. (1987). Molecular characterization of the oligopeptide permease of *Salmonella typhimurium*. *J Mol Biol* 195, 125-142.
- Kanagawa, K., Negoro, S., Takada, N. & Okada, H. (1989). Plasmid dependence of *Pseudomonas* sp. strain NK87 enzymes that degrade 6-aminohexanoate-cyclic dimer. *J Bacteriol* 171, 3181-3186.
- Kanagawa, K., Oishi, M., Negoro, S., Urabe, I. & Okada, H. (1993). Characterization of the 6-aminohexanoate-dimer hydrolase from *Pseudomonas* sp. NK87. *J Gen Microbiol* 139, 787-795.
- Kato, K., Ohtsuki, K., Mitsuda, H., Yomo, T., Negoro, S. & Urabe, I. (1994). Insertion sequence IS6100 on plasmid pOAD2, which degrades nylon oligomers. *J Bacteriol* 176, 1197-1200.
- Kinoshita, S., Negoro, S., Muramatsu, M., Bisaria, V. S., Sawada, S. & Okada, H. (1977). 6-Aminohexanoic acid cyclic dimer

hydrolase: a new cyclic amide hydrolase produced by *Achromobacter guttatus* KI72. *Eur J Biochem* **80**, 489–495.

Kinoshita, S., Terada, T., Taniguchi, T., Takene, Y., Masuda, S., Matsunaga, N. & Okada, H. (1981). Purification and characterization of 6-aminohexanoic acid oligomer hydrolase of *Flavobacterium* sp. KI72. *Eur J Biochem* **116**, 547–551.

Mori, H., Kondo, A., Ohshima, A., Ogura, T. & Hiraga, S. (1986). Structure and function of the F plasmid genes essential for partitioning. *J Mol Biol* **192**, 1–15.

Negoro, S., Shinagawa, H., Nakata, A., Kinoshita, S., Hatozaki, T. & Okada, H. (1980). Plasmid control of 6-aminohexanoic acid cyclic dimer degradation enzymes of *Flavobacterium* sp. KI72. *J Bacteriol* **143**, 238–245.

Negoro, S., Taniguchi, T., Kanaoka, M., Kimura, H. & Okada, H. (1983). Plasmid-determined enzymatic degradation of nylon oligomers. *J Bacteriol* **155**, 22–31.

Negoro, S., Kakudo, S., Urabe, I. & Okada, H. (1992). A new nylon oligomer degradation gene (*nylC*) on plasmid pOAD2 of *Flavobacterium* sp. *J Bacteriol* **174**, 7948–7953.

Okada, H., Negoro, S., Kimura, H. & Nakamura, S. (1983). Evolutionary adaptation of plasmid-encoded enzymes for degrading nylon oligomers. *Nature* **306**, 203–206.

Okazaki, M., Uemura, T., Nishimori, Y., Wakamatsu, K., Shimosaka, M., Saito, H., Negoro, S. & Okada, H. (1988). Analysis of a promoter-like region in *Flavobacterium* which controls 6-aminohexanoic acid linear oligomer hydrolase gene expression in *Escherichia coli*. *J Ferment Technol* **66**, 489–494.

Pearson, W. R. & Lipman, D. J. (1988). Improved tools for biological sequence analysis. *Proc Natl Acad Sci USA* **85**, 2444–2448.

Perego, M., Higgins, C. F., Pearce, S. R., Gallagher, M. P. & Hoch, J. A. (1991). The oligopeptide transport system of *Bacillus subtilis* plays a role in the initiation of sporulation. *Mol Microbiol* **5**, 173–185.

Rudner, D. Z., LeDeaux, J. R., Ireton, K. & Grossman, A. D. (1991). The *spo0K* locus of *Bacillus subtilis* is homologous to the oligopeptide permease locus and is required for sporulation and competence. *J Bacteriol* **173**, 1388–1398.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.

Tabata, S., Hooykaas, P. J. & Oka, A. (1989). Sequence determination and characterization of the replicator region in the tumor-inducing plasmid pTiB6S3. *J Bacteriol* **171**, 1665–1672.

Thomas, C. M. & Smith, C. A. (1986). The *trfB* region of broad host range plasmid RK2: the nucleotide sequence reveals *incC* and key regulatory gene *trfB/korA/korD* as overlapping genes. *Nucleic Acids Res* **14**, 4453–4469.

Tobin, M. B., Fleming, M. D., Skatrud, P. L. & Miller, J. R. (1990). Molecular characterization of the acyl-coenzyme A: isopenicillin-N acyltransferase gene (*penDE*) from *Penicillium chrysogenum* and *Aspergillus nidulans* and activity of recombinant enzyme in *Escherichia coli*. *J Bacteriol* **172**, 5908–5914.

Tsuchiya, K., Fukuyama, S., Kanzaki, N., Kanagawa, K., Negoro, S. & Okada, H. (1989). High homology between 6-aminohexanoate-cyclic-dimer hydrolases of *Flavobacterium* and *Pseudomonas* strains. *J Bacteriol* **171**, 3187–3191.

Vogelstein, B. & Gillespie, D. (1979). Preparative and analytical purification of DNA from agarose. *Proc Natl Acad Sci USA* **76**, 615–619.

Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.

Yomo, T., Urabe, I. & Okada, H. (1992). No stop codons in the antisense strands of the genes for nylon oligomer degradation. *Proc Natl Acad Sci USA* **89**, 3780–3784.

Zhou, Z. E., Nishimori, Y., Shimosaka, M. & Okazaki, M. (1990). Transcription of a gene for 6-aminohexanoic acid linear oligomer hydrolase of *Flavobacterium* sp. KI72 in *Escherichia coli*. *J Ferment Bioeng* **70**, 266–268.

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