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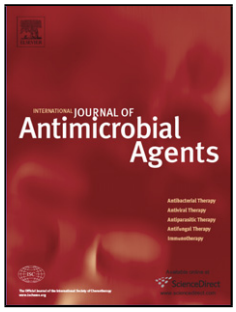

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Class 1 integrons in environmental and clinical isolates of *Pseudomonas aeruginosa*

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ABSTRACT

The aims of this study were to ascertain the presence and spread of class 1 integrons among environmental and clinical isolates of *Pseudomonas aeruginosa* and to characterise their variable regions. A total of 76 isolates (56 clinical and 20 environmental) were studied. The presence of plasmids was explored, and polymerase chain reaction (PCR) was used for integron detection. All amplicons were sequenced. PCR detected class 1 integrons in 26 of the 56 clinical isolates; environmental isolates were integron-free. No plasmids were found, thus all the integrons found are possibly on the chromosome. Most isolates presented one amplicon, except PA110514 and PA116136, which showed two PCR products each. Variable regions revealed that 18 strains carried only one gene involved in aminoglycoside resistance, whereas in 3 strains gene cassettes were not found. The most prevalent cassettes among isolates were those encoding aminoglycoside adenyltransferase B (*aadB*). Several of the strains had acquired the same or a highly similar cassette array as those detected in geographically distant *P. aeruginosa.* This finding suggests that contact with bacterial reservoirs contributes to the evolution of this pathogen towards multiresistance. Empty structures found may represent a reservoir increasing the capacity to adapt to the environment. However, these integrons are not retained when the selective pressure disappears. It is hypothesised that integrons containing gene cassettes are crucial vehicles for the rapid horizontal transfer of resistance. If this is so, reduced use of antibiotics may lead to a significant decrease in the carriage of integrons among *P. aeruginosa* strains.

1. Introduction

The opportunistic pathogen *Pseudomonas aeruginosa* is noted not only for its metabolic versatility and its exceptional capacity to adapt to and colonise a wide variety of ecological environments (water, soil, rhizosphere, animals), but also for its intrinsic resistance to a broad range of antimicrobial agents. Infections by *P. aeruginosa* are often difficult to treat because of its virulence and the relatively limited availability of effective antimicrobial agents. Nosocomial infections caused by *P. aeruginosa* frequently occur in ventilated and immunocompromised patients in Intensive Care Units [1,2]. Opportunistic infections caused by this pathogen can result from immunosuppression, which enhances the virulence of strains with low pathogenicity. Several studies have addressed the structure of *P. aeruginosa* populations [3–5].

Multiresistance of *P. aeruginosa* has been extensively described and involves components such as outer membrane proteins (OMPs), β-lactamases and other antibiotic-modifying enzymes, and efflux pumps. In the course of a population study of *P. aeruginosa* isolated in the hospital setting, we explored the basis of the resistance shown by this pathogen.

Increasing attention has been given to integrons in the context of resistance to antimicrobials. In particular, much research effort has been devoted to integrons belonging to classes 1, 2 and 3, the classes most commonly associated with the spread of antibiotic resistance in pathogens. Whilst integrons generally pose a problem for the management of spread of resistance, they are especially common in pathogenic bacteria [6]. Surveys examining the prevalence of various genetic

elements in multidrug-resistant strains invariably show a high correlation between the presence of a class 1 integron and particular antimicrobial resistance profiles. Most notable among these is resistance to ampicillin and streptomycin, and particularly to trimethoprim and sulfamethoxazole (SMX) [6,7]. In the case of class 1 integrons, SMX resistance is normally derived from *sul1*, which is located in a region downstream of the integron called the 3'-conserved segment (3'-CS), a region present in most class 1 integrons isolated from clinical environments [8,9]. Consequently, SMX resistance is a common feature of strains that carry class 1 integrons. The class 1 integrons that carry drug resistance genes in clinical isolates have a relatively conserved structure, which commonly comprises two conserved DNA sequences (5'-CS and 3'-CS) separating a variable region where mobile gene cassettes are located [10]. This arrangement has been used as a polymerase chain reaction (PCR) tool for the simple recovery of cassette arrays irrespective of knowledge of the many and varied cassettes that may be present [11,12]. In addition to recovering and analysing cassettes from specific isolates, PCR has also been used as a tool in broader epidemiological studies [13]. However, this method has some limitations. First, some class 1 integrons from clinical isolates do not have part or all of the 3'-CS, or alternatively they may carry a very large array of cassettes, thereby preventing detectable amplification. Either outcome can generate a false-negative result. In addition, PCR array length analysis alone underestimates cassette diversity since some array combinations may be similar or even identical in length [14]. Also, given that lateral gene transfer is mediated by a number of types of elements, the combinatorial exchange and spread of integron cassette arrays can occur in a variety of ways, including homologous recombination, transposition and even non-integron-mediated site-specific recombination. Thus, research into integron

cassette arrays in the absence of context may not give a truly accurate picture of the processes that influence the spread of resistance genes in pathogenic bacteria [9].

Here we detected and characterised the integrons carried by a group of *P. aeruginosa* isolates of clinical and environmental origin [5].

2. Materials and methods

2.1. Bacterial strains

A total of 56 clinical and 20 environmental *P. aeruginosa* isolates were studied. Clinical strains were isolated by the Servei de Microbiologia of the Hospital Universitari de Bellvitge (Barcelona, Spain). Environmental isolates were obtained from samples of water and fomites in the hospital setting, as described elsewhere [5]. All bacteriological media were obtained from Scharlab, S.L. (Barcelona, Spain).

2.2. Minimum inhibitory concentration determination

Antimicrobial susceptibility testing was performed using the microdilution method in Mueller–Hinton broth following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [15,16]. The following antimicrobials were tested: piperacillin; piperacillin/tazobactam; ticarcillin; aztreonam; gentamicin; tobramycin; amikacin; trimethoprim/sulfamethoxazole; ciprofloxacin; ofloxacin; ceftazidime; cefepime; meropenem; imipenem; and ampicillin. Antibiotics were purchased from Sigma Chemical Co. (St Louis, MO), except for cefepime, which was from Bristol-Myers Squibb (Rueil-Malmaison, France). For some strains, the disk diffusion method with Mueller–Hinton agar was also performed. Disks were purchased from

Oxoid Ltd. (Basingstoke, UK). Strains were classified as susceptible or resistant following the CLSI guidelines [15].

2.3. Plasmid isolation

Two strategies were used to obtain plasmids from the strains. In the first, conventional plasmids were detected by the alkaline lysis method described by Feliciello and Chinali [17]. Briefly, cells were lysed with sodium hydroxide/sodium dodecyl sulphate (NaOH/SDS), followed by precipitation of the cell lysate with 2 M potassium acetate/1 M acetic acid, and precipitation of the resulting supernatant with isopropanol. Finally, the precipitate was treated with RNase and was once again precipitated with isopropanol. The second method, for detecting megaplasmids, consisted of pulsed-field gel electrophoresis (PFGE) of genomic DNA using the method described by Barton et al. [18]. Briefly, bacteria were embedded in agarose gel and were lysed using a rapid protocol. After this, plugs were incubated with S1 nuclease (Fermentas, Vilnius, Lithuania) and were subjected to PFGE in agarose gels in a CHEF-DR III unit (Bio-Rad, Hercules, CA) for 14 h at 14 °C and 6 V/cm with pulse times of 45 s, and for 6 h at 14 °C and 6 V/cm with pulse times of 25 s. Electropherograms were visualised by ethidium bromide staining as described elsewhere [5].

2.4. Detection and analysis of integrons

DNA extraction for integron detection was performed by a modification of the method described by Lévesque et al. [11]. Briefly, strains were grown overnight in 10 mL of brain–heart infusion (Scharlab, S.L.) with 10% glycerol in the presence of a selective

antibiotic at 37 °C. After diluting the culture 1:5 with distilled water, the bacterial suspension was boiled for 10 min and was then centrifuged at 12 000 × *g* for 2 min. The supernatant was used for PCR analysis. PCR was carried out in a 100 μL volume containing 250 μM dNTP (Fermentas), 2.5 pmol of each primer (5'CS, 5'-GGCATCCAAGCAGCAAG-3'; and 3'CS, 5'-AAGCAGACTTGACCTGA-3') (Invitrogen, Camarillo, CA), 1× PCR buffer, 3 mM $MgCl_2$, 1 U *Taq* DNA polymerase (Fermentas) and 30 μL of freshly prepared bacterial suspension. Amplification was achieved as follows: initial denaturation at 94 °C for 12 min, followed by a three-step profile of 94 °C for 1 min, 55 °C for 1 min and 5 min of extension at 72 °C for a total of 35 cycles; 5 s were added to the extension time at each cycle. A final extension at 72 °C was carried out for 5 min and following this step all completed reactions were maintained at 4 °C. All reactions were performed in a GeneAmp® PCR System 2700 thermal cycler (Applied Biosystems, Foster City, CA). PCR products were electrophoresed at 100 V for 1.30 h on a 0.7% (w/v) Tris–borate–EDTA buffer (TBE) agarose gel and viewed by ethidium bromide staining as described elsewhere.

2.5. DNA sequencing and analysis of sequence data

Sequence data for the amplicons were obtained with primers 5'CS and 3'CS. For isolates PA110514, PA116136, P18 and P4, it was necessary to design additional internal primers in order to amplify variable regions of integrons [for cassettes InA1/InP18, primers 5'-A18L (GTGCAGAGAATGATCAGC), 5'-A18L2 (CCTCCACATCGTGGAA) and 5'-A18LR (TCTGTGGCGATGCACCA); and for cassette InP4, primer 5'-A4L (CTCCATAAGGTCATTGAGCA)]. PCR products were purified with a MinElute® PCR Purification Kit (QIAGEN, Crawley, UK) prior to

sequencing. An ABI PRISM BigDye® Terminator v.3.1 Cycle Sequencing Ready Reaction Kit and ABI PRISM® 3700 DNA Analyzer were used (Applied Biosystems). Database searches were conducted using the basic local alignment search tool (BLAST) [19].

3. Results and discussion

The genetic relationship between some of the isolates studied was analysed previously both by PFGE and polyacrylamide gel electrophoresis (PAGE) of OMPs showing a first cluster including four strains (three of clinical and one of environmental origin), a second cluster that also included clinical and environmental isolates, as did a third cluster.

An additional cluster with a high degree of diversity was formed only by environmental isolates, although distance suggested a poor relationship among them. Thus, measurement of genetic diversity by PAGE of OMPs disagrees with results from PFGE. Assuming that the outer membrane constituted a permeability barrier with a key role in antibiotic resistance, only some OMP combinations were expected to survive in hospitalised patients. Therefore, in this case selective pressure acted in the opposite manner, leading to lower diversity among clinical isolates, although genetic diversity as measured by PFGE was higher in this group [5].

Susceptibility to antimicrobials as percentage resistant, intermediate and susceptible strains is shown in Table 1. When the presence of plasmids was explored either by alkaline lysis or S1-PFGE, all strains gave negative results. Thus, although we

cannot confirm that these isolates are plasmid-free, it is feasible that the presence of plasmids is rare, which suggests that all the integrons found are on the chromosome.

The search for class 1 integrons by PCR using primers 3'CS and 5'CS, which led to the amplification of only the variable region (gene cassettes), resulted in the detection of class 1 integrons in 26 of the 56 clinical isolates.

Environmental strains were free of resistance and were completely integron-free. This observation may be a consequence of the lower capacity of these strains to acquire integrons or may simply imply that they do not interact with integron-bearing organisms. The prevalence of class 1 integrons among isolates decreases significantly as their origin lies progressively further from human influence. We thus conclude that antibiotics exert the main pressure that selects for and maintains the integrons.

Among integrons, class 1 integrons have received the greatest attention. They have been proposed to be largely involved in the dissemination of antibiotic or disinfectant resistance among clinical isolates; in fact, ca. 75% of clinical isolates carry at least one integron of this class [8,9].

The largest amplicon (3000 bp) was found in strain P4, whereas in P1 and P18 amplicons of ca. 2500 bp were detected, whilst isolates cc, cd and 28 gave amplicons of ca. 1000 bp. Isolates 7, 19, 22, 30, 33, 69, 76, 77, 87, 93, 99, cm, cn, co and B' had integrons giving amplicons of ca. 750 bp. Isolates cb, 17 and 26 showed the smallest amplicons (ca. 200 bp). Finally, PA110514 and PA116136 (an

imipenem-resistant derivative of PA110514) showed two PCR products in each strain, thereby suggesting the presence of two integrons in each; the amplicon lengths corresponding to the sizes of the variable regions were 2500 bp and 400 bp. Gene cassettes were not detected in all the integrons (Table 2).

Direct sequencing of the variable region revealed that 18 strains carried only one resistance gene involved in aminoglycoside resistance. The most prevalent cassettes among isolates were those encoding aminoglycoside adenyltransferase (*aad*).

The cassette most commonly found in integrons was *aadB* (strains 7, 19, 22, 30, 33, 69, 76, 77, 87, 93, 99, cm, cn, co and B'). This cassette encodes for the aminoglycoside-2'-*O*-adenyltransferase that confers resistance against kanamycin, gentamicin and tobramycin. *aadA7* was present in three strains (cc, cd and 28) and encodes for aminoglycoside adenyltransferase conferring resistance to streptomycin and spectinomycin. Sequencing revealed a high prevalence of genes conferring resistance to streptomycin and spectinomycin in the integrons. The former is used in clinics (e.g. to treat tuberculosis) and the second has been widely used as a growth promoter in food-producing animals. These uses thus result in an increase in selective pressure [20,21].

Sequencing of amplicons from strains P1, P4, PA110514, PA116136 and P18 revealed that the variable region contained a cassette array with various open reading frames (ORFs). The cassette array of P1 had three ORFs, all involved in aminoglycoside resistance; *aadB* encoding aminoglycoside-2'-*O*-adenyltranferase

that confers resistance to kanamycin, tobramycin and gentamicin; and *aadA11* encoding aminoglycoside-3'-adenyltransferase that confers resistance to streptomycin and spectinomycin. The third, named *orfE*, encodes an aminoglycoside-2''-adenylyltransferase that belongs to the conserved bacterial family pfam10706 and superfamily cl11303. The proteins included in these two families are involved in resistance to kanamycin, gentamicin and tobramycin and remove the synergism between aminoglycosides and cell wall-active agents. This cassette array also occurs in the integron In2345 (AY758206) of *P. aeruginosa* strain PA2345, isolated in the University Hospital of Besançon (France) [22], and both integrons share 100% identity for *aadB* and *orfE* and 98% for *aadA11*. Furthermore, the high similarity between sequences from bacterial isolates from different geographic regions suggests frequent global and cross-species spread of this cassette complex.

Strain P18 contained a cassette array with three ORFs, *orfL, tetR* and *orfJ*, in that order, and its variable region was identical to that of the largest amplicon present in strains PA110514 and PA116136. *orfL* encodes a phage integrase-like protein with an identity of 79% with the protein already described in plasmid pKLC102 [23]. This *orf* is overlapped with *tetR,* which belongs to the TetR family of transcriptional regulators involved in the transcriptional control of multidrug efflux pumps, pathways for the biosynthesis of antibiotics, response to osmotic stress and toxic chemicals, control of catabolic pathways, differentiation processes and pathogenicity [24]. Cassettes are preferentially integrated adjacent to the *attI1* site, but recombination events do not always occur as predicted (for example because of unusual recombination between an *attI* site and an *attC* site), thus creating a potential fusion of two adjacent gene cassettes [25]. *orfJ* encodes a hypothetical protein with

conserved domains belonging to the family pfam06977 and to the superfamily cl06158. These two families represent a conserved region ca. 100 residues long within a number of hypothetical bacterial proteins that may be regulated by SdiA, a member of the LuxR family of transcriptional regulators. *orfJ* is inserted in an inverted position with regard to the promoters, thus there may be no transcription of the putative protein.

The smallest amplicon present in strains PA110514 and PA116136 was an integron that lacked integrated gene cassettes but contained 5'-CS and 3'-CS, like the one in strains cb, 17 and 26. These empty structures may represent a reservoir that could confer bacteria the capacity to adapt rapidly to the environment by means of the acquisition of antibiotic resistance genes, among others, thus allowing selective advantage. Alternatively, the lack of integrated genes cassettes may be a consequence of the excision of previously integrated cassettes from the integron when antibiotic selective pressure is diluted in the environment, thus integrons could be in contact with, and even be acquired by, any strain, but not retained when selective pressure disappears. Recently it has been demonstrated that the expression of gene cassettes is regulated by an SOS response control [26] and antibiotic resistance genes can be silenced at no biological cost until they are required. However, these 'empty' structures appear to be indicators of the absence of sustained antimicrobial pressure.

The integron carried by P4 (Fig. 1), designated InP4 from its gene content, included *aacA4*, also called *aac(6')-II* (aminoglycoside 6'-*N*-acetyltransferase), downstream of the *attI1* recombination site, directly followed by the β-lactamase gene bla_{P1b} coding

for PSE-1/CARB-2, and the adenyltransferase gene *aadA2*. The AAC(6')-II family of enzymes provide resistance to tobramycin, netilmicin, kanamycin and gentamicin. AAC(6')-II is not only the most common AAC(6') but also the most common AAC in *P. aeruginosa* and is thus a significant determinant of gentamicin and tobramycin resistance in this organism. bla_{P1b} coding for PSE-1/CARB-2 carbenicillinase confers resistance to the β-lactams piperacillin, carbenicillin and ticarcillin. The last gene, *aadA2*, encodes for aminoglycoside-3''-*O*-adenyltransferase that confers resistance to streptomycin and spectinomycin. It has been argued that the common use of β-lactams and aminoglycosides for the clinical treatment of humans has contributed to the simultaneous presence of gene cassettes encoding β-lactamases and aminoglycoside-modifying enzymes in the same integron. Furthermore, the genes of this cassette, and also the complete cassette array, show high sequence identity (99%) with strains from distant countries such as China (*P. aeruginosa* strain PA466, GenBank accession no. FJ817423.1), Portugal (*P. aeruginosa* In99 and In100; GenBank accession nos. DQ219465.1 and AY560837.1 [27]) and Italy [28], among others.

The observation that geographically diverse *P. aeruginosa* strains have acquired the same or a highly similar cassette array suggests that contact with yet unidentified bacterial reservoirs contributes to the evolution of this pathogen towards multiresistance in Europe. This contribution is possibly by means of horizontal transfer of the complete integron structure. Antibiotics are used on a large scale in other ecological niches, for instance in animals for food production. In this context, these antibiotics may also contribute as sources or reservoirs of integrons carrying resistance genes [29].

Moreover, several studies have examined normal commensal microbiota from apparently healthy people, and high resistance rates to several antimicrobial agents have been observed. Thus, these bacteria could act as a reservoir for drug resistance genes recruited by pathogens under antibiotic pressure [30].

Integrons that contain gene cassettes provide a powerful vehicle for the rapid horizontal transfer of resistance across bacterial populations and thus could contribute to the sudden increase in the prevalence of multidrug-resistant infections in a community. The distribution of identical genes in organisms isolated from people living in disparate geographic regions indicates that they were more likely infected by organisms already harbouring such gene cassettes that originate in non-human reservoirs. That is, there appears to be a global ‘epidemic’ of mobile drug resistance genes, possibly spread by globalisation of trade.

The variety of structures found among class 1 integrons after more than half a century of antibiotic usage bears testament to the genetic flexibility and adaptability of the bacterial genome under environmental stress, characteristics that make these microorganisms ultimate survivors.

In conclusion, we propose that class 1 integrons are transient elements that foster antibiotic resistance in clinical environments, but not in the absence of antibiotic selective pressure. If this were the case, a decrease in antibiotic usage may lead to a significant reduction in the carriage of integrons among *P. aeruginosa* strains.

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Competing interests

None declared.

Ethical approval

Not required.

Nucleotide sequences accession nos.

The nucleotide sequences reported in this paper have been submitted to GenBank databases. The accession nos. for all the sequences are: In141, HM367607; In361, HM367608; InB, HM367609; Inl7, HM367610; Inl8, HM367611; Inl9, HM367612; Inl13, HM367613; Inl19, HM367614; Inl22, HM367615; Inl28, HM367616; Inl30, HM367617; Inl33, HM367618; Inl34, HM367619; Inl35, HM367620; Inl69, HM367621; Inl76, HM367622; Inl77, HM367623; Inl87, HM367624; Inl93, HM367625; InP18, HM367626; Inl99, HM367627; InP1, HM367628; and InP4, HQ157204.

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Fig. 1. Schematic representation of the cassette array contained in the variable region of the integron found in *Pseudomonas aeruginosa* P4. Inserted gene cassettes are indicated by grey arrows. The arrows show the direction of transcription of genes.

Table 1

Susceptibility of 76 *Pseudomonas aeruginosa* isolates to the antimicrobial agents tested

Susceptibility	% of isolates									
	TZP	CAZ	FEP	ATM	IPM	MEM	GEN	TIC	AMP	CIP
Susceptible	80.3	51.3	56.6	60.5	40.8	65.8	48.7	57.9	82.9	53.9
Intermediate	11.8	26.3	31.6	7.9	5.3	5.3	11.8	2.6	7.9	1.0
Resistant	7.9	22.4	11.8	31.6	53.9	28.9	39.5	39.5	9.2	45.1

TZP, piperacillin/tazobactam; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam; IPM, imipenem; MEM, meropenem; GEN, gentamicin; TIC, ticarcillin; AMP, ampicillin; CIP, ciprofloxacin.

Table 2

Characteristics of integrons and their gene cassettes identified in the integron-carrying clinical isolates

Isolate	Amplicon length (bp)	Integron	Cassette(s)	Protein encoded
Cc	1000	In*l*8	*aadA7*	Aminoglycoside adenyltransferase
Cd	1000	In*l*9	*aadA7*	Aminoglycoside adenyltransferase
28	1000	In*l*28	*aadA7*	Aminoglycoside adenyltransferase
7	740	In*l*7	*aadB*	Aminoglycoside-2'-*O*-adenyltransferase
Cm	740	In*l*13	*aadB*	Aminoglycoside-2'-*O*-adenyltransferase
Cn	740	In*l*34	*aadB*	Aminoglycoside-2'-*O*-adenyltransferase
Co	740	In*l*35	*aadB*	Aminoglycoside-2'-*O*-adenyltransferase
19	740	In*l*19	*aadB*	Aminoglycoside-2'-*O*-adenyltransferase
22	740	In*l*22	*aadB*	Aminoglycoside-2'-*O*-adenyltransferase
30	740	In*l*30	*aadB*	Aminoglycoside-2'-*O*-adenyltransferase
B'	740	InB	*aadB*	Aminoglycoside-2'-*O*-adenyltransferase
33	750	In*l*33	*aadB*	Aminoglycoside-2'-*O*-adenyltransferase
69	750	In*l*69	*aadB*	Aminoglycoside-2'-*O*-adenyltransferase
76	750	In*l*76	*aadB*	Aminoglycoside-2'-*O*-adenyltransferase
77	750	In*l*77	*aadB*	Aminoglycoside-2'-*O*-adenyltransferase
87	750	In*l*87	*aadB*	Aminoglycoside-2'-*O*-adenyltransferase
93	750	In*l*93	*aadB*	Aminoglycoside-2'-*O*-adenyltransferase
99	750	In*l*99	*aadB*	Aminoglycoside-2'-*O*-adenyltransferase

cb	200	Inl2	No gene	–
17	200	In1l7	No gene	–
26	200	Inl26	No gene	–
P1	2500	InP1	*aadB*	Aminoglycoside-2'-*O*-adenyltransferase
			orfE	Aminoglycoside-2''-adenylyltransferase
			aadA11	Aminoglycoside-3'-adenyltransferase
P18	2500	InP18	*orfL*	Putative phage integrase
			tetR	TetR
			orfJ	Putative protein, function unknown
P4	3000	InP4	*aac(6')-II*	Aminoglycoside 6'-*N*-acetyltransferase
			bla_{P1b}	β-Lactamase
			aadA2	Aminoglycoside-3''-*O*-adenyltransferase
PA110514	2500	In141	*orfL*	Putative phage Integrase
			tetR	TetR
			orfJ	Putative protein, function unknown
	400	In142	No gene	–
PA116136	2500	In361	*orfL*	Putative phage integrase
			tetR	TetR
			orfJ	Putative protein, function unknown
	400	In362	No gene	–