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Two Proteins with Ornithine Acetyltransferase Activity Show Different Functions in *Streptomyces clavuligerus:* Oat2 Modulates Clavulanic Acid Biosynthesis in Response to Arginine

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The *oat2* gene, located in the clavulanic acid gene cluster in *Streptomyces clavuligerus*, is similar to *argJ*, which encodes *N*-acetylornithine:glutamic acid acetyltransferase activity. Purified proteins obtained by expression in *Escherichia coli* of the *argJ* and *oat2* genes of *S. clavuligerus* posses *N*-acetyltransferase activity. The kinetics and substrate specificities of both proteins are very similar. Deletion of the *oat2* gene did not affect the total *N*-acetylornithine transferase activity and slightly reduced the formation of clavulanic acid under standard culture conditions. However, the *oat2* mutant produced more clavulanic acid than the parental strain in cultures supplemented with high levels (above 1 mM) of arginine. The purified *S. clavuligerus* ArgR protein bound the arginine box in the *oat2* promoter, and the expression of *oat2* was higher in mutants with a disruption in *argR* (arginine-deregulated), confirming that the Arg boxes of *oat2* are functional in vivo. Our results suggest that the Oat2 protein or one of its reaction products has a regulatory role that modulates clavulanic acid biosynthesis in response to high arginine concentrations.

Streptomyces clavuligerus produces clavulanic acid (CA), an important β-lactamase inhibitor synthesized by the condensation of arginine (23, 27), and glyceraldehyde-3-phosphate to form carboxyethylarginine (12, 18). In wild-type strains, arginine is a limiting precursor for clavulanic acid biosynthesis (23). Studies of the arginine gene cluster have been highly interesting as a way of understanding the channeling of this precursor into clavulanic acid (20, 21).

The presence of a cyclic arginine pathway in *Streptomyces* species has been reported previously (20). The *S. clavuligerus* arginine gene cluster contains an *argJ* gene encoding a protein of 39,733 Da, similar to other ArgJ proteins, as deduced from the genome sequences of *Streptomyces coelicolor* (74.9% identical amino acids), *Mycobacterium tuberculosis* (47.8% identity), and *Corynebacterium glutamicum* (40.7% identity). ArgJ recycles the acetyl group from *N*-acetylornithine to L-glutamic acid in the first step of the pathway. The *argJ* gene complements an *Escherichia coli* XSD2 mutant lacking *N*-acetylornithinase activity, indicating that the ornithine *N*-acetyltransferase (OAT) activity encoded by *argJ* is able to complement the *N*-acetylornithinase activity encoded by the *argE* gene of the lineal arginine pathway in *E. coli*.

In the clavulanic acid gene cluster of *S. clavuligerus*, the *oat2* gene (initially described as ORF6), located downstream of the *pah* gene, encodes a protein of 41,607 Da with a high similarity to ornithine acetyltransferases (9). ArgJ and Oat2 share 31.1% amino acid identity over the entire sequence. When *oat2* was subcloned and transformed into *E. coli* XSD2, the *argE* mutation of this strain was complemented, indicating that the *oat2* promoter was expressed in *E. coli*; moreover, the transformants showed a strong OAT activity but no ornithinase activity (20). A putative ARG box, which is characteristic of genes that are regulated by ArgR, is present upstream of *oat2* (nucleotides [nt] −90 to −52 upstream of *oat2*). This sequence has 64% identity with the consensus ARG box sequence of *Streptomyces* (21).

Due to the similarity of the *argJ* and *oat2* genes and to the presence of a putative arginine-controlled regulatory sequence upstream of *oat2*, it was important to clarify the role of *oat2* in the context of arginine and clavulanic acid biosynthesis. For this work, we have purified and characterized the proteins encoded by both *argJ* and *oat2*. Additionally, mutants of *S. clavuligerus* with deletions in *argJ* or *oat2* were obtained, and we demonstrate that the ARG box upstream of *oat2* is functional, acting as an arginine sensor. The Oat2 product appears to act as a modulator of clavulanic acid biosynthesis. A third paralogous ornithine acetyltransferase gene (named *oat1*) was very recently reported by Tahlan et al. (26) for *S. clavuligerus*.

MATERIALS AND METHODS

Biologicals. *S. clavuligerus* ATCC 27064, the producer of clavulanic acid, was used as a DNA source. *S. clavuligerus argR*::*neo* (20) was used as a host to detect *oat2* expression. *E. coli* DH5α and *E. coli* XL1-Blue were used for subcloning purposes and for the expression of heterologous proteins. *E. coli* pGST (Pharmacia) plasmid vectors were used for the expression of heterologous proteins. The cell extracts of *E. coli* pGEX-J and pGEX-O6 cultures induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 45 min were used for chromatography through a glutathione-Sepharose column.

E. coli-Streptomyces bifunctional pHZ1351-derived plasmids were used for gene disruption in *Streptomyces*. The pHZ1351 plasmid is unstable and integrates into the genome (13). The mutants obtained in this work were grown in SA (starch, asparagine) medium (16) supplemented with arginine when required, as previously described (18), or in TSB medium (Trypticase soy broth) when RNA isolation was required.

Antibiotic determination. Clavulanic acid and alanylclavam were quantified by a previously described bioassay (19, 22). Additionally, clavulanic acid was derivatized with imidazole and analyzed by high-performance liquid chromatography (HPLC) (6).

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Nucleic acid manipulations. DNA digestion, subcloning, and hybridization and *Streptomyces* transformation were performed as described by Sambrook et al. (24) and Kieser et al. (13).

argJ was amplified by PCR with pKS211 as a template and with the oligonucleotides argJ1 (5′-AGATCTCGAGCTGTACGCGCTGTTCT-3′) and argJ2 (5′-AACAGCTATGACCATG-3′) as primers. In the case of *oat2*, the template plasmid was pBS41 and the oligonucleotides used were oat2-1 (5′-AGATCTG GACATAGCCCTCGGTGA-3′) and oat2-2 (5′-AACAGCTATGACCATG-3′). The underlined sequences in the oligonucleotides correspond to BglI sites that were required to introduce the amplified fragment in frame. DNA amplification was obtained in 25 cycles as follows: denaturation at 94°C for 45 s, followed by annealing at 55°C for 45 s and extension at 72°C for 1.5 min. The amplified fragments were isolated from agarose gels, digested with BglI, and subcloned into BglI-digested pGEX-3X to produce plasmids pGEX-O6 and pGEX-J, respectively.

Southern blot hybridization was performed in the presence of 50% formamide at 42°C, and the filters were washed twice at room temperature with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) and additionally at 65°C with the same solution. Gene disruption was performed as described by Pérez-Redondo et al. (17), using pHZ1351-derived plasmids.

Dot blot hybridization of total RNAs was done as described by Sambrook et al. (24). RNAs were isolated from TSB cultures and extracted as described by Pérez-Redondo et al. (17). Hybridization was performed by applying total RNAs (20 μg) to the wells of a Blot-Dot SF apparatus (Bio-Rad) equipped with a Hybond-N membrane (Amersham). The RNAs were fixed with a Stratalinker UV cross-linker and prehybridized overnight at 55°C in prehybridization solution (5× SSC, 5× Denhardt's solution, 0.5% SDS, 0.5 mg of salmon sperm DNA/ml, 50% formamide). The filters were washed at 25 and 65°C, hybridized with 5 ng of an [α-^{32}P]dCTP (3,000 Ci/mol)-labeled probe/ml at 58°C for 4 h, and then washed again twice. The hybridization signals were quantified in an Instant Imager (Packard Instruments, Meriden, Conn.).

Electrophoretic mobility shift assays (EMSAs) were performed as described by Santamarta et al. (25). A labeled probe (0.1 to 0.5 ng) and protein (0.2 to 5 μg) were placed in a final volume of 20 μl of buffer A (20 mM HEPES [pH 7.9], 4 mM Tris-HCl [pH 7.4], 50 mM KCl, 5 mM $MgCl_2$, 0.125 mM $MnCl_2$, 10% glycerol) in the presence of 1 μg of herring sperm DNA or poly(dI-dC) and then were incubated for 20 min at 25°C. The gel was prerun for 30 min, the samples were applied in 5 μl of developing buffer, and electrophoresis was carried out at 90 V for 4 to 6 h. Radioactivity was visualized as described above.

Protein analysis. For the detection of His-tagged or glutathione *S*-transferase (GST)-fused proteins, immunodetection protocols supplied by the manufacturers were used. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). When required, the proteins were blotted onto polyvinylidene difluoride (Immobilon-P, Millipore) membranes in a MiniTrans-Blot apparatus (Bio-Rad) at 90 V for 2 h as recommended by the manufacturer.

Purification of ArgR. ArgR was purified as described by Czaplewski et al. (4), with the following modifications. A mycelium of *S. clavuligerus* grown for 48 h in SA medium was disrupted by sonic treatment in a B12 sonifier (Branson Sonic Power). After centrifugation at 18,100 × *g* for 15 min, the pellet was suspended in Arg buffer (25 mM Tris-HCl, 10 mM $MgCl_2$, 0.5% phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, pH 7.5), solid NaCl was added to 0.5 M, and the mixture was incubated for 20 min at 30°C. After centrifugation at 18,100 × *g* for 30 min at 4°C, the supernatant was extensively dialyzed against Arg buffer supplemented with 75 mM NaCl. The pellet formed in the dialysis bag was recovered by centrifugation and suspended in Arg buffer containing 0.5 M NaCl. This protein was diluted with an equal volume of Arg buffer and applied to an *S*-Sepharose ion-exchange column equilibrated with Arg buffer–250 mM NaCl, and the protein was then eluted with a 250 to 800 mM NaCl gradient. The sequence of the N-terminal end of ArgR was determined in a 476A Applied Biosystem Sequencer.

Ornithine acetyltransferase activity. Measurements of ornithine acetyltransferase activity (EC 2.3.1.35) and quantification with acid ninhydrin solution (1% ninhydrin in methanol–0.4 M citric acid [2:1]) of the amount of ornithine formed were performed as described by Denes (5). Alternatively, the acetylated products were measured by HPLC analysis according to the method of Marc et al. (14), using a μBondapack C_{18} (3.9 by 300 mm) column and 0.1 M phosphoric acid–methanol (9:1) as a liquid phase at a flow rate of 1 ml/min. Acetylated compounds in the eluted fractions were measured at 210 nm and compared with plots of pure standards. Under these conditions, the retention time for *N*-acetylglutamic acid was 4.2 min, that for *N*-acetylarginine was 4.1 min, and that for *N*-acetylcysteine was 7.2 min. One unit of ornithine *N*-acetyltransferase is the amount of enzyme that forms 0.01 μmol of ornithine per min at 37°C.

RESULTS

Characterization of ArgJ and Oat2: processing of the proteins. In order to obtain information about the role of *argJ* and *oat2* on arginine and clavulanic acid biosynthesis, we purified the proteins encoded by both genes as fusion products with GST as indicated in Materials and Methods. The ArgJ and Oat2 proteins were well expressed; cell extracts showed OAT activities in the range of 60 to 80 U/mg of protein, an activity that was absent in untransformed *E. coli* XL1-Blue cells. ArgJ and Oat2 were purified by affinity chromatography through glutathione-Sepharose and were analyzed by SDS-PAGE. Two major protein bands, of about 45 kDa (product of the fusion of GST to the α subunit of ArgJ or Oat2) and 22.5 kDa (hereafter named the β subunit), were found in the purified fractions containing ArgJ or Oat2, but bands close to the 67-kDa size for the expected GST-ArgJ or GST-Oat2 fusion proteins were never found. After thrombin digestion, only the 45-kDa protein was found to be excised, yielding a 17.2-kDa protein. Abadjieva et al. (1) reported that *Saccharomyces cerevisiae* ornithine acetyltransferase is an autoprocessing enzyme. To detect if the same phenomenon occurs in the ArgJ and Oat2 proteins of *S. clavuligerus*, we isolated the 22.5-kDa bands (putative β subunits) from the gels and determined their N-terminal sequences. Sequences TLLTFFA (amino acids 181 to 187) and TLLVVL (amino acids 179 to 184) were obtained from the Oat2 and ArgJ bands, respectively, purified from the gel. These results indicate that autoprocessing occurs in both cases at a threonine residue, which agrees with the hypothesis of Marc et al. (15) and with the result described by Kershaw et al. (11) for Oat2 while this work was in progress. Both proteins appear to belong to the N-terminal nucleophile hydrolase class of self-processing acetyltransferases (3).

Molecular masses of ArgJ and Oat2 and evidence for the formation of α-β heterotetramers. In order to determine the molecular masses of the native proteins, we analyzed freshly prepared proteins by fast-performance liquid chromatography in a Superose HR6 (Pharmacia) column with 50 mM Tris-HCl (pH 7.5). Under these conditions, Oat2 showed a molecular mass of 87.2 ± 4.5 kDa, which correlates well with the theoretical value of 83.2 kDa and corresponds to a heterotetramer containing two α and two β subunits derived from processing of the original protein. The ArgJ heterotetramer showed a size of 76.8 ± 3.5 kDa, which is in agreement with the size of 79.4 kDa deduced from the sequence of the gene.

Kinetics of processing of ArgJ and Oat2. Purified GST-ArgJ and GST-Oat2 preparations were incubated at 30°C in the presence or absence of amino acids (1 mM) that are known to affect autoprocessing (8). An analysis by SDS-PAGE indicated that Oat2 was completely self-processed after 45 min of incubation at 30°C, while the autoprocessing of ArgJ was less efficient. In both cases, processing was activated by cysteine, while arginine and glycine were partially inhibitory and alanine, lysine, serine, threonine, and ornithine did not affect processing.

Comparative optimal parameters of ArgJ and Oat2. The optimal pHs for both enzymes were tested in 25 mM phosphate buffer (pHs 4.5 to 7.5) and 100 mM Tris-HCl buffer (pHs 7.5 to 9.5). The transacetylation activity was optimal in the range of pHs 7.0 to 9.5 for both enzymes, with a maximum

TABLE 1. Characteristics of OAT activities of ArgJ and Oat2[a]

Protein	V_{max} (U/mg of protein)	K_m for *N*-acetylornithine (mM)	K_m for glutamic acid (mM)	K_i for ornithine (mM)
ArgJ	2.32	4.23	17.32	0.69
Oat2	5.70	4.47	25.11	0.93

[a] The optimal pH for both proteins was 8.5, and the optimal temperature was 37°C.

value at pH 8.5 (Table 1). The effect of temperature on transacetylation activity was tested in the range of 4 to 45°C. The optimal temperature for both enzymes was 37°C, with 80% activity at 45°C and <20% activity below 20°C. K_m and V_{max} values were calculated by a colorimetric assay and the HPLC method, with similar results. The K_m and V_{max} for *N*-acetylornithine and glutamic acid were in the same range for both enzymes (Table 1). A Lineweaver-Burk representation of the ArgJ and Oat2 activities on increasing concentrations of glutamic acid (1.5 to 9 mM) and *N*-acetylornithine (1.5 to 9 mM) indicated a ping-pong BiBi-type reaction. These parameters are in the same range as those found for ornithine acetyltransferases of *Bacillus stearothermophilus*, *Methanococcus jannaschii*, or *Thiobacillus neopolitans* for *N*-acetylornithine and glutamic acid for ArgJ (14) and as those reported by Kershaw et al. (11) for Oat2.

Inhibition of the enzymes by L-ornithine was studied by HPLC, which analyzed the formation of acetylated compounds. Ornithine behaved as a competitive inhibitor of glutamic acid for both enzymes. On the other hand, ornithine was a mixed inhibitor of *N*-acetylornithine in the case of Oat2 and a noncompetitive inhibitor for ArgJ, with both types of inhibitions being compatible with a ping-pong BiBi-type reaction. The K_i values for Oat2 and ArgJ were 0.69 and 0.93 mM, respectively.

Substrate specificity. Neither ArgJ nor Oat2 was able to use acetyl-coenzyme A as a donor to acetylate the different amino acids that were tested as substrates. Acetylated amino acids such as *N*-acetylaspartate, *N*-acetylalanine, or *N*-acetylleucine did not support the acetyltransferase reaction. *N*-Acetylcysteine produced traces of acetyl-glutamic acid (5%) with both enzymes, and *N*-acetyllysine (a structural analogue of *N*-acetylornithine) gave acetyltransferase activity (38%) with the pure Oat2 or pure ArgJ (30%) enzyme. The best acetyl donor (100%) in the reactions was *N*-acetylornithine.

The preferred acceptor substrate for acetylation catalyzed by ArgJ or Oat2 with *N*-acetylornithine as an acetyl donor was glutamic acid (100%). Aspartic acid, leucine, alanine, and α-aminoadipic acid were not used as acceptors. A low acetyltransferase activity was found with arginine as an acceptor, with an efficiency of 13% by the Oat2 protein and of 11% by the ArgJ protein. In summary, both ArgJ and Oat2 show relatively similar substrate amino acid specificities, and both of them appear to be involved primarily in the acetylation of glutamic acid by using *N*-acetylornithine as a donor. The Oat2 protein does not support the acetyltransferase reaction that is required to form holomycin from holothin (7).

Disruption of *argJ* and *oat2* and its effect on ornithine acetyltransferase activity. In order to determine the in vivo role of ArgJ and Oat2 in clavulanic acid biosynthesis, we constructed

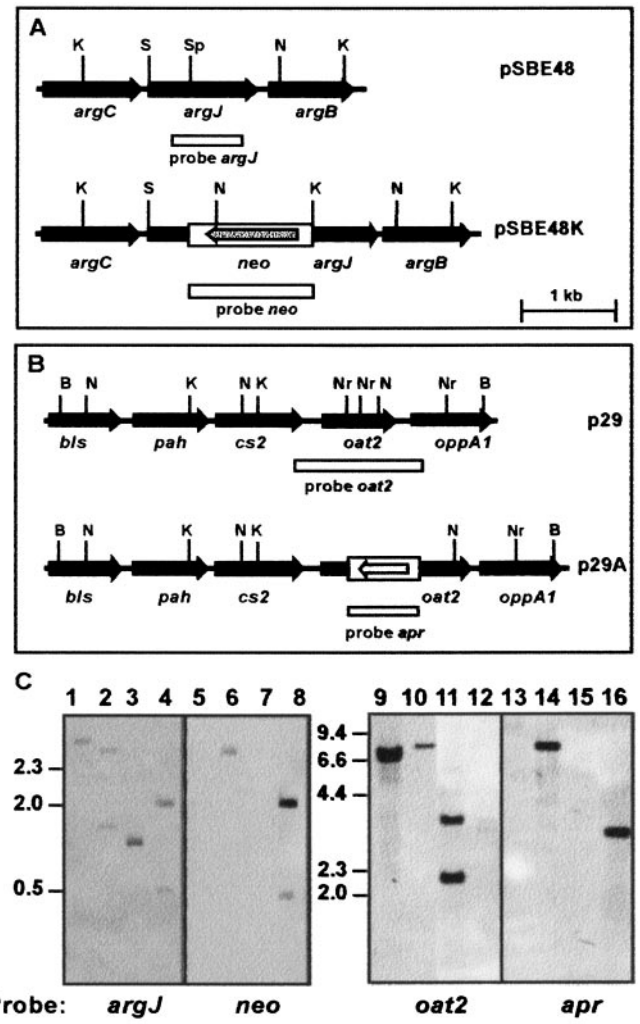

FIG. 1. Scheme of disruption of *argJ* (A) and *oat2* (B) showing the restriction sites for KpnI (K), SacI (S), SphI (Sp), NcoI (N), BglII (B), and NruI (Nr). The probes used in the hybridization experiment to confirm the correct disruption are shown below. (C) Hybridization with an *argJ* probe (lanes 1 to 4), a *neo* probe (lanes 5 to 8), an *oat2* probe (lanes 9 to 12), and an *apr* probe (lanes 13 to 18). The lanes show the digestion of *S. clavuligerus* total DNA with BglII (lanes 1 and 5), SacI and NcoI (lanes 3 and 7), BglII (lanes 9 and 13), and NcoI (lanes 11 and 15); of *S. clavuligerus argJ*::*apr* total DNA with KpnI (lanes 2 and 6) and with SacI and NcoI (lanes 4 and 8); and of *S. clavuligerus oat2*::*apr* total DNA with BglII (lanes 10 and 14) and NcoI (lanes 12 and 16).

mutants of *oat2* and *argJ*. For the deletion of *oat2*, an internal 531-bp NruI DNA fragment from *oat2* in plasmid p29, which contains the *bls*, *pah*, *cs2*, *oat2*, and *oppA* genes, was removed and replaced with the *apr* [*aac*(3)*IV*] gene for apramycin resistance (Fig. 1). For the disruption of *argJ*, plasmid pSBE48 carrying the *argCJB* genes of *S. clavuligerus* was digested with SphI, which cuts 154 nt downstream of the start codon of *argJ*, and the kanamycin resistance *neo* gene (previously named *aph*) was inserted. These constructs were subcloned as XhoI-XbaI fragments in the *Streptomyces* vector pHZ1351 and were used to transform *S. clavuligerus*. The *S. clavuligerus* mutants were selected for apramycin resistance-thiostrepton sensitivity (*oat2* insertion-deletion mutant) or for kanamycin resistance-thio-

TABLE 2. OAT activity in the wild type and in mutants of *S. clavuligerus*[a]

S. clavuligerus strain	OAT activity (mU/mg of protein)	% of wild-type activity
27064	826 ± 28	100
argJ::*neo*	633 ± 37	76
Δ*oat2*::*apr*	889 ± 62	107
argJ::*neo* Δ*oat2*::*apr*	575 ± 32	69

[a] Cell extracts of cells were grown in TSB medium for 36 h. Data are averages from triplicate assays.

strepton sensitivity (*argJ* disruption mutant), as described by Pérez-Redondo et al. (17). The deletions-insertions were confirmed by hybridization with a SacI-BstXI probe internal to *argJ* and the whole *neo* gene in the case of the *S. clavuligerus argJ*::*neo* mutant and with a PvuI probe containing the *oat2* gene and a DraI-EcoRI probe containing the *apr* gene in the case of the *S. clavuligerus* Δ*oat2*::*apr* mutant (Fig. 1B). A double mutant, *S. clavuligerus argJ*::*neo* Δ*oat2*::*apr*, was derived from *S. clavuligerus argJ*::*neo* by the use of a p29-derived plasmid for *oat2* gene disruption.

The specific OAT activities in *S. clavuligerus* and the mutant strains were measured in extracts from 36-h cultures in TSB medium (Table 2). The disruption of *argJ* reduced the acetyltransferase activity 24%. However, the OAT activity of the *oat2* insertion-deletion mutant remained unaltered, suggesting that the OAT activity observed for purified Oat2 is of little relevance to the total OAT cellular activity. Moreover, after the disruption of *argJ* and *oat2*, 69% of the total OAT activity still remained in the cell extracts, indicating that other uncharacterized enzymes might additionally carry out the acetyltransferase reaction in *S. clavuligerus*.

The *argJ*::*neo* mutant was auxotrophic for arginine, apparently due to the inactivation of expression of the arginine genes downstream of *argJ*. This mutant produced holomycin at the same levels as the wild-type strain.

Production of clavulanic acid by strains with *oat2* and *argJ* disruptions and the effect of arginine on production. The *S. clavuligerus* Δ*oat2*::*apr* mutant consistently produced about 50% of the clavulanic acid in TSB medium that the wild-type strain did and 40% of that by the wild type in defined SA medium (Fig. 2).

The production of clavulanic acid in complex TSB medium by the mutant with an *argJ* disruption was in the same range as that of the wild-type strain, indicating that *argJ* disruption has little effect on CA biosynthesis; this strain was unable to grow properly in defined SA medium due to its auxotrophy, as it required at least 0.1 mM arginine for growth. Therefore, we took advantage of the arginine requirement of the *argJ* mutant to compare the effect of increasing amounts of arginine (0.1 to 20 mM) on clavulanic acid production by the wild-type strain and the *argJ* and *oat2* mutants in SA medium. Under these growth conditions, wild-type *S. clavuligerus* and the mutant with an *argJ* disruption showed a clear dose-response relationship. Low arginine concentrations (below 1 mM) stimulated clavulanic acid production, but the levels of clavulanic acid at 2.5 to 20 mM arginine were on the order of 40 to 60% of those in cultures with 1 mM arginine. Interestingly, clavulanic acid production by *S. clavuligerus* Δ*oat2*::*apr* was not as strongly affected by arginine (2.5 to 20 mM) as that of the wild type; in the mutant, there was little or no regulation by high concentrations of arginine, and therefore the yields of clavulanic acid were 150 to 230% of those found in the wild-type strain (Table 3). Furthermore, this mutant produced about 200% more alanylclavam in SA medium than the wild-type strain. This value increased 20% when the medium was supplemented with 10 mM arginine. Therefore, it seems that the regulation of clavulanic acid biosynthesis by high arginine concentrations is mediated by the *oat2* gene.

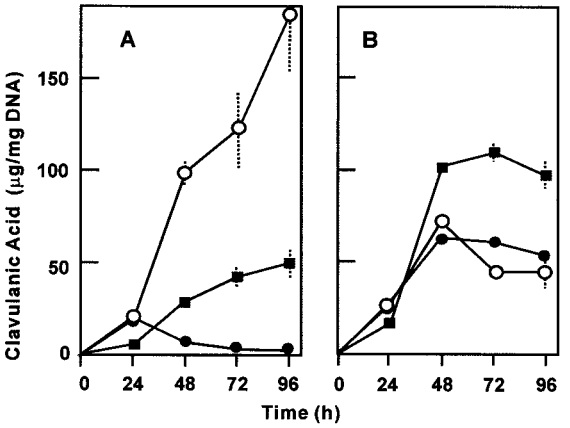

FIG. 2. Effect of arginine on clavulanic acid production in SA medium. Data are shown for *S. clavuligerus* 27064 (○), *S. clavuligerus argJ*::*neo* (●), and *S. clavuligerus* Δ*oat2*::*apr* (■). (A) 0.1 mM arginine; (B) 10 mM arginine. Note that the lack of production of clavulanic acid by *S. clavuligerus argJ*::*neo* in the presence of 0.1 mM arginine is related to poor growth.

The regulatory ARG box upstream of *oat2* is functional. A putative ARG box, GTGCATAAATTTGCCACTCTATGGG

TABLE 3. Production of clavulanic acid by *S. clavuligerus* 27064 and *oat2*::*apr* in SA medium supplemented with increasing amounts of arginine[a]

Incubation time (h)	*S. clavuligerus* strain	Amt of clavulanic acid (μg/mg of DNA [% of the yield with 1 mM arginine supplementation]) in presence of indicated concn of arginine (mM)[b]						
		0	0.5	1	2.5	5	10	20
48	27064	166 (68)	160 (66)	243 (100)	119 (49)	118 (48.5)	143 (59)	132 (54)
	oat2::*apr*	66.4 (26)	48 (19)	251 (100)	186 (74)	212.4 (84)	238.3 (95)	277.2 (110)
72	27064	130 (58)	142 (63)	225 (100)	84 (37)	85 (37)	105 (47)	88 (39)
	oat2::*apr*	48.4 (21)	54.3 (23)	229.4 (100)	142.5 (62)	158.3 (69)	247 (107)	151.8 (66)

[a] Cultures were grown in triplicate flasks, and the experiment was repeated three times. The standard deviations varied between 5 and 10%.
[b] Arginine was added to the cultures at time zero.

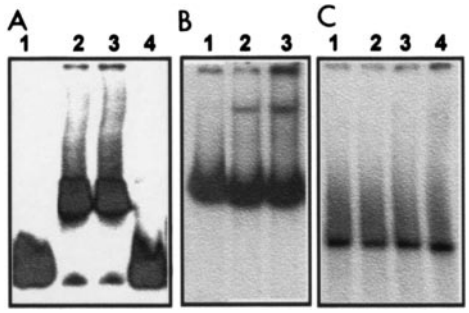

FIG. 3. Gel shift electrophoresis of a 354-bp DNA fragment containing the *argC* promoter (A), a 368-nt DNA fragment obtained by PCR containing the *oat2* promoter of the clavulanic acid gene cluster (B), and a 714-bp DNA fragment containing the *cefD-cmcI* bidirectional promoter of the cephamycin C gene cluster (C). Lanes 1, free probes; lanes 2 and 3, EMSAs with the ArgR-containing fraction eluting at 600 mM NaCl (20 and 40 ng of protein, respectively) from the *S*-Sepharose column; lanes 4, EMSAs with the fraction eluting from the *S*-Sepharose column at 400 mM NaCl (40 ng of protein), which does not contain ArgR, as a negative control.

AAATAATGCAGA, is present upstream of *oat2* (9, 20). However, the functionality of this regulatory region is unknown. To test whether the ArgR protein controlling the arginine regulon was able to bind the *oat2* regulatory region, we purified the ArgR protein from *S. clavuligerus* by ion-exchange chromatography in an *S*-Sepharose column as described above. The ArgR-containing fractions were tested by EMSAs using three DNA fragments containing the following different regions: (i) a 354-bp XbaI-XhoI DNA fragment containing the promoter of *argC*, which is known to have two ARG boxes in tandem (20); (ii) a 306-bp XbaI-XhoI DNA fragment containing the *oat2* ARG box; and (iii) a 714-bp DNA fragment containing the bidirectional *cefD-cmcI* promoter, which contains the site of binding for the unrelated CcaR protein (25) and was used as a negative control. EMSAs (Fig. 3) showed that fractions eluting at 600 mM NaCl from the *S*-Sepharose column produced a clear shift of the *argC* promoter (Fig. 3A, lanes 2 and 3) and of the *oat2* promoter regions (Fig. 3B, lanes 2 and 3), but not of the *cefD-cmcI* promoter-containing fragment (Fig. 3C, lanes 2 and 3). Competition studies using the unlabeled *oat2* probe indicated that the effect was specific (not shown). The purified fractions obtained by elution from the *S*-Sepharose column with 600 mM NaCl were dialyzed and analyzed by SDS-PAGE. They contained an isolated protein band with an apparent size of 24 kDa by SDS-PAGE. The N-terminal end of the band was determined to be MARH(N/R)I, which was identical to the sequence deduced from the *argR* gene, MARHRI, thus confirming that we were dealing with the ArgR-containing fraction. The difference in the size found by SDS-PAGE for ArgR (expected size, 17 kDa) might be explained by the very basic nature of this protein. The sequence found for the N-terminal end of the protein confirmed that the translation initiation given by Rodríguez-García et al. (21) was the correct one. The shift shown in Fig. 3 indicates that the arginine box present upstream of *oat2* is a binding site for ArgR and that this gene is effectively part of the arginine regulon; therefore, the expression of *oat2* is likely to be down-regulated by high concentrations of arginine.

Expression of *oat2* in wild-type *S. clavuligerus* and in a mutant with an *argR* disruption. In order to test whether the gel shift produced by ArgR for the promoter region of *oat2* resulted in positive or negative regulation of expression of the gene, we hybridized the total RNAs from *S. clavuligerus* ATCC 27064 (wild type) and *argR*::*neo* cultures grown for 12, 24, and 36 h in TSB medium with a 0.5-kb NruI probe internal to *oat2*. As a positive control, a probe containing the autoregulated *argR* gene was used (20). The results shown in Fig. 4 indicate a strong hybridization of the *oat2* transcript band with the total RNA of the *S. clavuligerus argR*::*neo* strain compared with that of the wild type, indicating that the expression of *oat2* is under negative control by ArgR. When they were quantified, the levels of mRNA that were specific for *oat2* were 4.9-, 6.9-, and 5.5-fold higher than those of the wild-type strain at 12, 24, and 36 h of culture. The strong RNA hybridization shown in Fig. 4 when the *argR* probe was used also confirms that the *argR* gene is negatively autoregulated.

DISCUSSION

The *S. clavuligerus* ArgJ and Oat2 ornithine acetyltransferases show very similar in vitro kinetic constants and optimal pHs and temperatures. Both ArgJ and Oat2 are processed at an internal threonine (AT sequence) and appear to have a heterotetrameric $\alpha_2\beta_2$ structure. In this regard, both enzymes

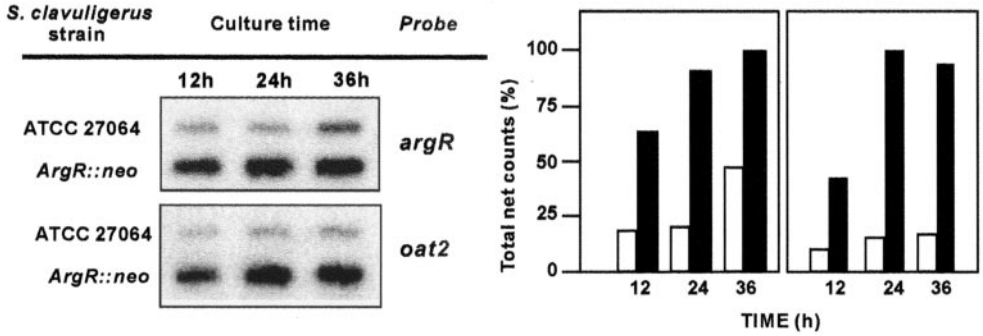

FIG. 4. Comparative expression levels of *oat2* and *argR*. (Left) Slot blot hybridization of 8 μg of RNA from *S. clavuligerus* 27064 and *S. clavuligerus argR*::*neo* grown in TSB medium for 12, 24, and 36 h with the following probes: a 483-nt probe obtained by PCR and containing the whole *argR* gene and a 0.5-kb NruI DNA fragment internal to *oat2*. (Right) Densitometric analysis of hybridization. The slot corresponding to the hybridization at 36 h of culture (32,500 cpm for *argR* and 17,000 cpm for *oat2*) was considered to be 100%.

belong to the N-terminal nucleophile hydrolase enzyme family (3), for which the N-terminal residue of the β subunit (usually threonine) is involved in the active center.

It is interesting that the mutant with an *argJ* disruption is auxotrophic, despite the presence of other OAT activities in *S. clavuligerus* that contribute about 70% of the total OAT activity in cell extracts. The arginine auxotrophy of the mutant with an *argJ* disruption might reflect a polar effect on the transcription of arginine biosynthesis genes located downstream of *argJ*.

The *oat2* gene is able to be expressed in *E. coli* from its own promoter and complements the *argE* auxotrophy of *E. coli* XS1D2. It also confers OAT activity, but not ornithinase activity, to the transformants (20). However, the deletion of *oat2* did not decrease the OAT activity in *S. clavuligerus* cell extracts, indicating that this protein does not contribute to the OAT activity on glutamic acid in vivo and that Oat2 probably has an in vivo role that is different from that of ArgJ and is most likely related to the control of clavulanic acid biosynthesis (see below). Recently, a paralogous gene for *oat2*, *oat1*, has been found in a separate location in the *S. clavuligerus* genome (26). It is unclear, however, whether Oat1 contributes OAT activity to *S. clavuligerus* cell extracts.

Oat2 has a clear positive role in clavulanic acid production since the *oat2*-negative mutant produces only about 35% of the amount of β-lactamase inhibitor that the wild type produces (Fig. 2A). In addition, disruption of the recently reported *oat1* gene partially reduces clavulanic acid production, and this effect is additive in *oat1 oat2* double mutants, especially in defined SA medium (26). Oat2 may contribute to channeling arginine to *N*-acetylarginine or glutamic acid to *N*-acetylglutamate under conditions of low arginine concentrations, probably with the synergistic action of Oat1, which explains the reduction of clavulanic acid production in mutants with an *oat* deletion. However, the acetylation of arginine by Oat2 occurs to a very small extent (13%) compared with the activity on glutamic acid. Moreover, the activity on arginine is not very different from that found for ArgJ (11%); therefore, these results do not support an arginine-channeling role for Oat2 and instead suggest a duplication of the channeling of glutamic acid (a substrate for many other competing enzymes) toward *N*-acetylglutamate and the late steps of the arginine-clavulanic acid pathway. Nothing is known about the ability of Oat1 to convert arginine to *N*-acetylarginine, and the additive action of both enzymes may have a more important channeling role. *N*-Acetylarginine has been reported to be a substrate of the clavaminate synthase (11). However, the kinetic mechanisms described for the β-lactam synthetase, the next enzyme in the pathway, do not support the use of *N*-acetylarginine as a substrate (2).

The most interesting difference between *argJ* and *oat2* is the distinct behavior of the mutations in each gene in relation to clavulanic acid biosynthesis. The clavulanic acid production of *S. clavuligerus argJ*::*neo* was similar to that of the wild-type strain as long as there was enough arginine for growth. The wild type and *S. clavuligerus argJ*::*neo* showed an identical negative regulation of clavulanic acid production by an excess of arginine at concentrations above 1 mM, in spite of the precursor role of this amino acid; the addition of 10 mM arginine resulted in a 50 to 60% reduction in clavulanic acid production. However, the clavulanic acid production by the *S. clavuligerus oat2* mutant in the presence of high arginine concentrations increased relative to that of the wild-type strain, at some times reaching the levels of the wild type in the absence of arginine. Similar results were observed for the production of alanylclavam, a branching metabolite of the clavulanic acid pathway (10). These results suggest that an Oat2 reaction product (or the protein itself) has a negative regulatory role on clavulanic acid at high arginine concentrations that is removed in the mutant with the *oat2* deletion.

Purified ArgR from *S. clavuligerus* acts on the *oat2* promoter region, producing a characteristic gel shift indicative of DNA binding, probably to the ARG box present in the *oat2* promoter. In most cases, ArgR binding to ARG boxes results in the repression of biosynthetic genes of the arginine regulon; however, derepression after ArgR binding has been observed for genes involved in arginine catabolism. This second possibility was excluded by the results for the hybridization of mRNAs from the wild type and an *S. clavuligerus* mutant with an *argR* disruption, which clearly showed that *oat2* expression is repressed by ArgR (Fig. 4).

In summary, Oat2 catalyzes in vitro the *N*-acetyltransferase reaction, the first step of the arginine pathway. Its first role might therefore be to contribute to an increased channeling of glutamic acid toward arginine and clavulanic acid, although the deletion of *oat2* did not reduce the global *N*-acetylornithine transferase activity in the cells. A second role of Oat2 is to negatively regulate clavulanic acid biosynthesis at high arginine concentrations. This action may be mediated by an Oat2 reaction product, e.g., *N*-acetylarginine might inhibit carboxyethylarginine utilization in the cyclization reaction by the β-lactam synthetase. We cannot exclude, however, that the Oat2 protein itself may have a direct regulatory role on clavulanic acid biosynthesis. The expression of the *oat2* gene is under negative regulation by the ArgR regulator, which fits well with its proposed role on arginine and clavulanic acid biosynthesis.

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